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THE MEGALOBLAST-NORMOBLAST PROBLEM: A CYTOLOGIC STUDY

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MINNEAPOLIS, MINN.

THE large collection of slides resulting from the sternal aspirations done at the Mayo Clinic in cases of pernicious anemia and on patients with some clinical or hematologic features suggestive of this disease gave us the opportunity for detailed cytologic studies that we hoped would solve some of the numerous problems associated with the characteristic pathologic picture of the marrow in this disease.

The general pathologic picture of this marrow during relapse and its normalization after specific treatment have been described by many authors with little difference of opinion. However, study of the detailed cytologic picture of marrow of pernicious anemia during complete relapse and in various phases of remission has resulted in many different interpretations of the types of cells present during relapse and beginning normalization, and of the origin and relations of these cells. Our study is devoted to these problems.

We have no intention of reviewing the extensive literature on marrow of pernicious anemia because this has been done by several authors. Extensive reviews have been published by Jones (1938, 1943[31-33]), Ferrata, Storti,[64, 65] Ferrata and Storti, Fieschi,[19, 20] Scott, Naegeli, Fallon, Limarzi and Levinson, Dacie and White, Rohr (1949),[54] Nordenson,[47, 48] Segerdahl, Chevallier,[6, 7] Bilski-Pasquier, and others. However, we do wish to review briefly a few of the significant papers which deal with the specific problems that have interested us.

Although the megalo-normoblast problem has been investigated and discussed ever since Ehrlich first proposed the name "megaloblast" for the large nucleated red cells of pernicious anemia, there are still a few questions concerning which there is no general agreement. Some still believe, with Ehrlich, that megaloblastosis is a reversion to the embryonic type of erythropoiesis and that megaloblasts are, therefore, identical with the primitive red cells of the embryo. However, since it has been shown that they differ from the latter in nuclear structure, basophilia of cytoplasm of the most primitive members of the series, chemistry and staining reaction of the hemoglobin, it seems that megaloblasts represent either a pathologic form of erythrocyte or a new generation of red cells which appear most frequently in the anemias due to deficiency of the liver factor. Both opinions are expressed in recent literature.

Some believe that the most immature megaloblasts (promegaloblasts) also function as the early progenitors of the normal series of developmental stages and are, therefore, to be found in small number in normal marrow. Normally they mature to normoblasts and

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erythrocytes, but in pernicious anemia their maturation is disturbed, causing many or most of them to mature to megaloblasts and megalocytes. In certain stages of the disease their maturation is blocked, resulting in the accumulation in the bone marrow of a large number of promegaloblasts, or of even more primitive forms, the erythrogones of some authors. This is one of the most characteristic features of the marrow of pernicious anemia during extreme relapse when the erythrocyte count is very low. According to this hypothesis the primitive cel's, the promegaloblasts or erythrogones, will again mature to normoblasts after liver therapy, or they and the more differentiated megaloblasts will gradually transform to normoblasts through a series of transitional stages.

Another view is that the most primitive forms of the red cell series are either promegaloblasts or pronormoblasts, and that there are no relations between the two except that both may be derived from myeloblasts. A modification of this hypothesis is the one which derives the megaloblasts from cells of the reticulum or undifferentiated mesenchyme and the normoblasts from myeloblasts. In these latter two hypotheses there is no room for a primitive ''erythrogone'' which, depending on conditions, can develop to either megaloblasts or normoblasts.

As Jones,[31, 33] Schartum-Hansen[56] and Schulten have suggested, difference of opinion concerning the origin and relations of megaloblasts is due in part to confusion of terminology rather than to differences of observation. Thus, some authors use the term ''megaloblast'' for immature red cells which others would include with the normoblasts of normal marrow. According to Schulten, further difficulty is due to the use of the name for erythroblasts which are more primitive than either promegaloblasts or pronormoblasts. He stated that these primitive cells may occur in any marrow showing erythropoietic hyperplasia with shift to the left. They should not be called megaloblasts or pronormoblasts until they definitely can be recognized as such, usually not until they have a trace of hemoglobin. They are the ''erythrogones'' of Helly, Ellermann, Dameshek and Valentine, Henning and Keilhack, and others. Schulten and Schartum-Hansen[56] prefer to name them ''proerythroblasts.''

Naegeli's ''proerythroblasts'' were young pronormoblasts (his makroblasts) with nucleoli. This is another illustration of the use of the same term for different cells. However, it is likely that Naegeli and Schulten were seeing similar cells, for Naegeli admitted it may be difficult to distinguish between proerythroblasts and promegaloblasts, although the former never mature to megalocytes.

A number of investigators have claimed the existence of a primitive basophilic cell, with leptochromatic nucleus and usually with irregular-shaped nucleoli, which functions as a stem cell for red cells, both normoblasts and megaloblasts, and which never develops to granulocytes or megakaryocytes. According to Schulten, this is the cell which, when interpreted as a megaloblast, has caused so much difference of opinion. If such a cell exists and if, as claimed, it occurs in hyperplastic marrow of pernicious anemia and a variety of other diseases, it can be assumed that it is at least in part responsible for the hypotheses of Pappenheim,[51] Sabin and Miller, Doan, Isaacs,[27, 28] Osgood and Ashworth, and others who see direct relations between megaloblasts and normoblasts.

Although Segerdahl (1935) used the name ''promegaloblast'' for the youngest red cells of pernicious anemia in relapse, she could not distinguish them from pronormoblasts. Her youngest red cells correspond, therefore, to the erythrogones of others, at least in theory. Henning and Keilhack (1939) were also unable to distinguish the youngest pronormoblasts from promegaloblasts. They used the term ''erythrogone'' for the youngest red cells of pernicious anemia in relapse. They seem to assume that this cell is a stem cell for both lines of red cells, and that the two lines cannot be distinguished until they have developed beyond the early ''pro'' stage. A similar concept of the relations of the two lines of red cells has been expressed by Grawitz, Barta, Jaffé, and Israëls.

Dameshek and Valentine (1937) have described and illustrated their primitive erythroblast in great detail. It is a very basophilic cell, generally large, with leptochromatic nucleus and irregular indistinct nucleoli. They stated that it is not confined to pernicious anemia marrow but may be found in any hyperplastic marrow with shift to the left in erythropoiesis. It is, therefore, an ''erythrogone'' which differs from myeloblasts and

reticular cells but seems to be derived from the latter. In their opinion megaloblasts and normoblasts constitute separate independent lines which, however, converge in the erythrogone.

Jones[31, 33, 34] and most other authors who believe in the independence of the two series have seen the myeloblast as the stem cell for the two lines, while others, like Storti (1937, 1939)[64, 65] and Ferrata and Storti (1948), have derived the megaloblasts from indifferent mesenchyme (reticulum) and normoblasts from myeloblasts. Jones found the myelob'ast to be the usual stem cell for the two series, but in some cases they may develop directly from reticulum without passing through the myeloblast stage. He did not recognize an erythroblast which serves as a common stem cell for both series. Barta, Fieschi (1940),[20] Ferrata and Storti, Schartum-Hansen,[56] Lambin and de Weerdt,[38] and Schleicher[59] also have expressed the belief that both lines may develop from the reticulum.

Several investigators have noted hyperplasia of reticulum in bone marrow in some cases of pernicious anemia. Among these may be mentioned Rohr,[54] Dameshek and Valentine, Scott, Markoff, and Schleicher.[59] Rohr,[54] Scott, and Dameshek and Valentine noted transformation of some of the free cells of the hyperplastic reticulum to promegaloblasts. Scott stated that many of the characteristics of the nucleus of the megaloblast are shared by that of the reticular cell. This was also noted by Haenel, who observed that this characteristic nuclear pattern is retained to some extent in the later developmental stages. In his opinion this results from the loss of power for heteroplastic division of the young reticular cells which, therefore, mature gradually with retention of some of their original nuclear characters. Jones (1943)[33] found that this type of nucleus can result from the reconstruction of the nuclear pattern after cells have passed beyond the myeloblast stage, so that it does not necessarily signify reticular origin of the cell. Jones saw a reticular origin for some megaloblasts, but expressed the belief that the majority are derived from myeloblasts. The early developmental stages resulting from this double origin of megaloblasts are illustrated on the colored plate of his section in Downey's *Handbook of Hematology*. Introzzi also emphasized the occurrence of two types of young megaloblasts having nuclear characters similar to those of the myeloblasts or reticular cells from which they originate. He presented a colored plate to illustrate the development from reticulum.

Berman, Jacobson and Vonder Heide expressed the belief that the deficiency state responsible for megaloblastosis may occur at the myeloblast level or that it may affect the reticulum, as claimed by Schleicher. Schleicher[59] derived both cell lines from the reticulum and expressed the belief that in pernicious anemia the reticulum of the marrow is involved in the pathologic process, and that it is this functionally altered reticulum which differentiates to promegaloblasts. Some normal erythrogenic reticulum remains as a source of pronormoblasts after specific treatment. In the marrow, therefore, the primary effect of the disease is on the reticulum, which results in the formation of pathologic granulocytes as well as red cells.

Some authors seem to regard only the earliest primitive erythroblasts as megaloblasts (Doan, Cunningham and Sabin; Isaacs;[27, 28] Osgood and Ashworth [Atlas]; Young and Osgood; Vogel, Erf and Rosenthal; Witts), and this may explain their finding megaloblasts in normal marrow and in that of various types of anemia. They do not seem to recognize the more mature megaloblasts of the marrow of pernicious anemia. For them, therefore, there is only one series of red cells, megaloblasts being the least mature members of the series.

For these investigators the transformation of megaloblastic to normoblastic marrow after effective treatment is a relatively simple matter and involves only the maturation of erythroblasts, which are the youngest members of the normal erythrocytic series (their megaloblasts). They do not have to postulate the presence of an "erythrogone" because this most primitive of the erythroblasts is a megaloblast which normally matures to a normoblast, according to their view, but fails to mature in a megaloblastic marrow.

Much study and speculation have been devoted to the dramatic changes which occur in the marrow of a pernicious anemia patient in severe relapse after the beginning of adequate treatment with liver extract, vitamin B_{12}, or folic acid. In some patients normoblastosis

may be quite marked as early as twenty-four hours after the first dose, and its beginnings may be seen even earlier. Interest has been centered on the exact origin of these normoblasts and on the fate of the megaloblasts, especially of the promegaloblasts, which disappear so rapidly as the normoblasts increase. It is generally agreed that some normoblasts are present in the marrow during relapse and before treatment, and that they proliferate rapidly soon after the beginning of effective treatment. However, proliferation of these scarce normoblasts does not seem sufficient to account for their rapid increase in number. This increase is explained in several hypotheses, usually based on their authors' views of normal and pathologic erythropoiesis. Heteroplastic development from myeloblasts accounts for the additional normoblasts according to Jones (1938).[32] In the opinion of Davidson, Davis, and Innes (1942) the rapid increase is due to direct transformation of early megaloblasts to pronormoblasts and basophilic normoblasts. Since they could not find a significant increase of mitoses during the early transformation period, they concluded that megaloblasts and normoblasts constitute a single line of erythroblasts which can change from one type to another in either direction.

A primitive erythrogone or proerythroblast is the precursor of most of the new normoblasts according to Dameshek and Valentine, Schulten, Henning and Keilhack, and Israël's among others. Since promegaloblasts are no longer developed from this cell, their number is rapidly reduced.

Haenel (1948) saw cells identical to the erythrogone in fair number in the marrow of untreated patients, but in his opinion they are exclusively stem cells of normoblasts whose development has been suppressed by the lack of the liver factor. Introduction of this factor results in rapid proliferation of the erythrogones and their differentiation to normoblasts. Megaloblasts are derived from reticulum, and some of the younger ones may transform to normoblasts during normoblastosis. Dameshek and Valentine, and Leitner also have expressed the belief that there is some transformation of megaloblasts to normoblasts during the early recovery period.

Fieschi (1940)[20] thought that some of the normoblasts, which increase so rapidly during this period, may be derived from his "hemohistioblasts" (hematopoietic reticular cells). He did not recognize an erythrogone and derived most of the normoblasts from "hemocytoblasts" (myeloblasts). In 1946, in a monograph with Astaldi, he claimed that in tissue culture of marrow of pernicious anemia, made shortly after administration of liver extract to the patient, there was rapid normalization of the culture and transformation of early megaloblasts to normoblasts. The older megaloblasts either died or completed their maturation. Similar observations were made by Astaldi, Baldini and Frugoni in 1948. However, they added the liver extract to the culture of marrow from untreated patients. It is claimed that basophilic megaloblasts transformed to basophilic normoblasts through a series of transitional stages, which are illustrated with photographs and a colored plate. These observations are important, even though it may not be agreed that the proliferative differentiation of basophilic megaloblasts results in the formation of true normoblasts. This will be discussed later in connection with our own observations.

Transformation of early megaloblasts to basophilic normoblasts, through a series of "intermediate erythroblasts" after treatment, had been described earlier, especially by Lambin and de Weerdt (1938, 1939),[38, 39] and by Koller (1939). A very detailed description of this transformation has been given by the former authors (1938). They stated that young megaloblasts of any degree of differentiation up to and including the polychromatophilic stage transform to intermediate cells of the same stage of development, and these in turn change to the corresponding normoblasts. Only a few megaloblasts mature. The "intermediate erythroblasts" were also seen to occur in the marrow of patients going into relapse before the anemia had become severe and before there were any megaloblasts in the marrow. Similar intermediate cells were also seen in hepatic cirrhosis and in infantile anemias. A good photograph of the intermediate erythroblasts made one day after the first administration of liver extract is presented by Koller on page 608, and by Rohr (1949)[54] in his Fig. 66. Mallarmé (1948) also described intermediate erythroblasts and stated that "In incipient cases the erythroblasts are not very much different from normoblasts, and there are inter-

mediary forms between normoblasts and megaloblasts.'' After treatment he saw rapid transformation of megaloblasts to normoblasts, but this involved only the youngest stages. In 1948, Bilski-Pasquier described intermediate erythroblasts and stated that they appear at about twenty hours after specific treatment.

Veeneklaas (1942) made a detailed analysis of the bone marrow of three infants who had megaloblastic anemia. Before treatment the marrow of all three was similar to that of pernicious anemia in relapse, and the response to treatment was similar to that which occurs in pernicious anemia. The change in size and in nuclear and cytoplasmic characters is illustrated in a series of Price-Jones curves, which show that the change to normal marrow is very gradual, and that the young megaloblasts become smaller and finally transform to normoblasts. A special type of ''intermediate cell'' is not mentioned, although the graphs prove that it was seen.

Zuelzer, Newhall and Hutaff (1947) also studied bone marrow of megaloblastic anemia in infants. In seven cases megaloblasts were present but the predominating cells were of intermediate types. In one patient there were intermediate cells but no megaloblasts at the first examination. The latter appeared as the anemia progressed without treatment for one month. The presence of intermediate cells without megaloblasts, therefore, was regarded as the first change of the erythroblasts during development of megaloblastic anemia, while the appearance of megaloblasts is a relatively late event. Twelve and twenty-four hours after treatment with folic acid the majority of the cells were of intermediate structure although megaloblasts, most of them acidophilic, were still present.

These authors concluded that treatment caused mitotic proliferation of the young megaloblasts, including the polychromatophilic forms, which resulted in the production of intermediate cells which gradually changed to normoblasts. Abnormal features which have been thought to be characteristic of megaloblasts were also seen in the intermediate cells. The older megaloblasts completed their maturation; they did not transform to intermediate cells because they were no longer capable of mitosis.

Davidson, Davis, and Innes (1942) saw no significant increase in the number of mitoses during treatment. From this they concluded that megaloblasts are changed directly to normoblasts. Photographs of their cells of types I to IV are supposed to illustrate this gradual change. However, the photographs do not show any cells corresponding to the ''intermediate cells'' of other authors.

In an extensive monograph Schartum-Hansen (1948)[57] claimed that there is only one series of erythroblasts which exist in various forms, depending on conditions. Many types of erythroblasts are present in the marrow of untreated pernicious anemia. Numerous intermediate cells prove that they all belong to a single series. The type which predominates depends on the amount of antipernicious anemia factor available. After treatment normal erythropoiesis develops gradually through transitional stages linking the types present during relapse with the developing normoblastic types. Megaloblasts are regarded as pathologic cells which degenerate before they reach the megalocyte stage.

A somewhat similar view of erythropoiesis in megaloblastic anemia has been expressed by Dacie and White (1949), but with a much simpler terminology. The various intermediate erythroblasts which result from deficient hemopoietic factors are grouped under the term ''intermediate megaloblasts'' because, in these authors' opinions, the degree of megaloblastic change depends on the amount of hemopoietic factor that is lacking, and so is proportional to the severity of the anemia. As seen in their photographs, cells that are more normoblastic than megaloblastic are included with the intermediate megaloblasts because, in their opinion, the pathogenesis of these cells is the same as that of more typical megaloblasts, the difference being merely quantitative and the cells less definitely abnormal. They expressed the belief that after treatment the maturing megaloblasts complete their development to megalocytes, while the most immature megaloblasts divide normally and differentiate to normoblasts. A very mixed picture results from the appearance of intermediate cells of varying degrees of abnormality if the liver extract is not fully potent. Additional literature on intermediate cells is discussed on their page 25.

It will be noted that the later conclusions of Schartum-Hansen (1948)[57] and of Dacie and White (1949) are quite similar to the views expressed by Zuelzer and associates in 1947.

Pappenheim (1912),[50] although he wrote before specific treatment was available, described intermediate cells that were impossible to classify. He believed that megaloblasts and normoblasts constitute separate lines and that megaloblasts do not transform to normoblasts. The latter opinion seems to have been changed later, for in his posthumous monographs edited by Hirschfeld (1919, 1920)[51, 52] a series of colored lithographs is presented to show the transformation by "differential proliferation" which results in the production of intermediate forms. This transformation is not confined to the youngest cells but includes all stages up to orthochromatic megaloblasts. Individual cells do not change from one type to another; the change is a gradual one through a series of mitoses resulting in the production of cells with intermediate nuclear pattern.

Israëls, and Jones (1943),[33] who do not believe in the transformation of megaloblasts, could not find, after treatment, any cells identical to the "intermediate erythroblasts" of Lambin and de Weerdt[38, 39] and others. Later Jones (1946)[34] described atypical megaloblasts, intermediate between megaloblasts and normoblasts, which appear at the beginning of therapy and in early relapse. Since these cells show a closer resemblance to megaloblasts than to normoblasts, they are considered to be the earliest form of megaloblastic regeneration. They were believed not to be related to normal erythropoiesis. The "intermediate megaloblasts" of Dacie and White were considered to be pathologic alterations of the normoblast series, rather than an independent line of cells derived from myeloblasts, as claimed by Jones.

Jones (1938)[31, 32] expressed the belief that megaloblasts disappear by degeneration of some and completion of maturation by the majority. Fieschi (1940)[20] stated that liver treatment prevents the formation of new megaloblasts at the promegaloblast level but does not interfere with the maturation of those already present. He saw little evidence for degeneration and that only in megaloblasts containing some hemoglobin. Storti (1937)[64] also expressed the belief that megaloblasts complete their maturation after treatment, but he did not see the increased rate of maturation described by Rohr[53] and Scott, although basophilic megaloblasts disappear rapidly. Scott saw an increase in the number of polychromatophilic megaloblasts while the promegaloblasts and basophilic megaloblasts were being reduced in number. However, Lambin and de Weerdt,[38] and Nordenson[48] claimed reduction in the number of older megaloblasts during the early recovery period. Only a few megaloblasts mature according to Lambin and de Weerdt,[38] and Leitner (1949). Henning and Keilhack stated that they do not all mature. They all degenerate according to Schartum-Hansen (1948).[57]

There is general agreement that the marrow becomes very active soon after specific treatment is begun, as shown by an increased number of mitoses and cells. There is some difference of opinion regarding the types of erythroblasts involved in this earliest activity. The first change is a greatly increased rate of proliferation of pronormoblasts and basophilic normoblasts according to Nordenson,[48] Storti (1937),[64] Rohr,[53] and Lambin and de Weerdt.[38] Some of these early normoblasts are always present according to these and other authors. Nordenson stated that some normoblasts resulting from the proliferation are atypical in that they show early formation of hemoglobin or persistent basophilia of cytoplasm. Rohr (1937)[53] found the basophilic normoblasts to be most numerous when the transformation of the marrow is slow; when rapid, the older stages are more numerous. During the early reactive period he found it difficult to distinguish between promegaloblasts and pronormoblasts. Lambin and de Weerdt noted very numerous basophilic normoblasts twenty hours after treatment was begun. The promegaloblasts had disappeared at this time. The proliferation responsible for the numerous basophilic normoblasts had not resulted in their reduction in size, from which Lambin and de Weerdt concluded that the mitoses must have been in still larger cells, and the majority of early megaloblasts must have transformed to normoblasts. In Scott's opinion the earliest marrow change is an increase in the rate of maturation of the cells of the megaloblastic series. He saw an increase in the polychromatic and orthochromatic megaloblasts before the increase of normoblasts took place.

Rohr gives his present opinion of megaloblast-normoblast relations in the second edition of his book, *Das menschliche Knochenmark*, 1949.[54] He describes and illustrates intermediate cells (Übergangsformen) which appear in pernicious anemia marrow during treatment. Some of these mature to large erythrocytes, while others, through a series of rapid divisions, transform to basophilic normoblasts. The dormant normoblastosis is stimulated at the same time. He and Schartum-Hansen[56, 57] noted slow or incomplete normalization of the marrow after inadequate doses of liver extract.

MATERIAL AND METHODS

Our study was confined to bone marrow obtained by sternal aspiration, done by Dr. G. L. Pease, from cases of hemolytic anemia and sprue, and from pernicious anemia in various stages of relapse, and during the first few hours following specific treatment with liver extract, folic acid, or combinations of the two, and vitamin B_{12}. In some cases of pernicious anemia, dosage was held to a minimum in order to prolong the transitional period and thus facilitate study of the cytologic changes which occurred.

For pernicious anemia the large collection of bone marrow slides of the Mayo Clinic was available, as well as bone marrow from patients who came to the clinic during the progress of this investigation. We also had slides of bone marrow from patients with pernicious anemia donated by Dr. Louis R. Limarzo and E. M. Schleicher. Schleicher also contributed slides of cases of pernicious anemia and hemolytic anemia prepared by his decolorizer technique,* which brings out the finest details of nuclear structure. Dr. R. Dorothy Sundberg contributed marrow from a case of acute hemolytic anemia following chicken pox. The Mayo Clinic material we used also included marrow from two cases of sprue with megaloblastic anemia, several cases of hemolytic anemia, and of normoblastic hyperplasia of unknown origin with left shift.

Most of the material studied was prepared by the following technique:

A small amount of heparin was added to the marrow immediately after aspiration. After gentle agitation smears were made from this unconcentrated marrow, and marrow particles were selected for sectioning and for imprints and spreads made from particles stuck to the ends of applicator sticks as suggested by Schleicher. A Wintrobe hematocrit tube was filled from the remaining marrow, which was then centrifuged. After the percentage volume of the layers of fat, plasma, nucleated cells (myeloid-erythroid layer), and erythrocytes had been recorded, the myeloid-erythroid layer, together with a small amount of plasma, was aspirated with a capillary tube and mixed. Smears were made from this concentrated marrow, dried rapidly, and stained with Wright's stain. This is essentially the method described by Schleicher,[58] and Schleicher and Sharp, and Limarzi (1947).[41]

For most of the forty-five patients studied we had smears of concentrated and unconcentrated marrow, and spreads made from the marrow particles. Usually the smears of the concentrated marrow were most favorable for our study, but in some cases the spreads from the particles showed the best cytologic detail. This was especially true in the few cases in which the centrifuging caused damage or distortion of cells. Usually the erythroid cells were undamaged even when monocytes, histiocytes, and mature neutrophils showed considerable distortion. For the few outside cases contributed for this study we had only films of concentrated marrow.

Since sections of the aspirated particles proved to be of little value for the problems that we wished to investigate, few of them were examined. Their chief value was for determining the degree of involvement of the reticulum in the pathologic alteration of the marrow.

In all cases of pernicious anemia, marrow was aspirated before treatment, and in all but eight cases there were one, two, and in a few cases three subsequent aspirations. Of

*Overstain in Wright's stain. Decolorize in a mixture of acetone 0.5 ml., methyl alcohol 5.0 ml., and distilled water 100 ml. For best results control under microscope.

chief interest for our problems were the thirteen cases in which the second aspiration was done at various intervals between six and twenty-four hours, and the cases in which marrow transformation was slow or incomplete because of the small dosage used in treatment.

OBSERVATIONS

A. Erythrogones or Procrythroblasts.—We have shown that several investigators of the problem of erythropoiesis believe in the existence of an erythroblastic stem cell which can differentiate to all types of erythrocytes encountered in normal and pathologic marrow. This is the "erythrogone" of Ellermann, Jaffé, Dameshek and Valentine, and Henning and Keilhack, or the "proerythroblast" of Schulten, Schartum-Hansen[56] and Israëls. As described it is a basophilic cell with nucleus and nucleoli suggesting origin of the cell from the reticulum, as claimed by several authors. However, it is more differentiated than the hematopoietic reticular cell (hemohistioblast) and can be identified as an exclusive erythroblastic stem cell that is more primitive than pronormoblasts or promegaloblasts. It is rarely encountered in normal marrow but may occur in fair number in left shift normoblastic hyperplasia. However, it is most numerous in pernicious anemia marrow during relapse. According to Schulten it is the "megaloblast" of authors who describe megaloblasts in normal marrow and in marrow of patients who have diseases not of the pernicious anemia type.

In our investigation we were interested in determining, to our own satisfaction at least, whether such a cell really exists or whether, like Segerdahl, the authors who have described it were unable to distinguish between pronormoblasts and promegaloblasts and, therefore, assumed the existence of a common erythroblastic stem cell for all types of red cells. As examples of the most immature erythroblasts in smears of aspirated bone marrow in cases of untreated pernicious anemia in relapse we refer to our Figs. 1 to 4, Plate I. All of the cells of this plate were photographed at a magnification of 800, and they are reproduced without reduction. Figs. 1 to 3 are from the same slide. The three cells shown in Fig. 4 are from another untreated patient.

It will be noted that there is considerable variation in size of the cells and that they tend toward irregularity of shape. The amount of cytoplasm relative to the nucleus is variable, but tends to be greater than is usually seen in the most immature erythroblasts which occur in normoblastic hyperplasia. The degree of basophilia of the cytoplasm is also quite variable, as is the distribution of the basophilic "spongioplasm" in the cytoplasm. The extremes in this respect can be seen by comparing Fig. 1 with the upper cell in Fig. 2 and the left-hand cell in Fig. 4. This difference in basophilia does not seem to depend entirely on the degree of differentiation, as can be seen by comparing the nuclei of these cells.

The nuclei of these primitive erythroblasts have an extremely delicate stippled chromatin pattern showing very uniform distribution of the chromatin granules. In the megaloblastic series this pattern is maintained for some time after differentiation begins, the chromatin granules becoming merely coarser and more irregular, as seen in the promegaloblast of Fig. 7, which is from a

patient having pernicious anemia forty-eight hours after treatment with folic acid began. Megaloblasts are still the predominant type of erythroblasts in this marrow. The cells of Figs. 2, 3, and 4 are more basophilic and have a slightly coarser nuclear pattern than the cells of Figs. 1 and 5, but the chromatin pattern is not quite as coarse as in the promegaloblast of Fig. 7. These variations may be due to slight differences in the degree of development toward megaloblasts, which would make cells 1 and 5 the most immature of the group. That the fine nuclear pattern and lack of cytoplasmic basophilia of cell 5 is not due to defective staining is shown by comparison with the cells of Fig. 7, which are from the same slide. The slide is one donated by Schleicher; it was treated with his decolorizer in order to bring out the finest nuclear details.

All of these cells have nucleoli which are extremely variable in size, shape, and intensity of staining. They may be well stained and sharply outlined as in Figs. 3 and 4, or they may be faint or barely visible as in Figs. 1 and 5. They may form one large unbroken irregular mass, or the cell may have several irregular-shaped nucleoli.

Many of the free cells of the reticulum (hemohistiocytes, hemohistioblasts, hematopoietic reticular cells) have nuclei and nucleoli similar to those of the primitive erythroblasts illustrated on our Plate I. This similarity of nuclei, and the hyperplasia of reticulum seen in many patients having untreated pernicious anemia, have led many investigators of the erythropoiesis problem to assume a reticular origin of the most immature megaloblasts (Ferrata, Storti,[64] Dameshek and Valentine, Henning and Keilhack, Rohr,[53, 54] Scott, Lambin,[37] Fieschi,[19, 20] Haenel).

The cells of our Figs. 1 to 5 give a fair sample of the types of primitive erythroblasts which we encountered in the marrow of patients having pernicious anemia. Cells 1 to 4 are from untreated patients who had practically no normoblasts in their marrow, so that it can be assumed that if the cells differentiated and the hematopoietic factor that was lacking was not replaced they would develop to megaloblasts. Cells 1 and 5 are the most primitive of the types shown. We interpret them as being intermediate between free reticular cells and the other cells illustrated on Plate I. Similar cells are not numerous, probably because heteroplastic development from the reticulum is of no great importance for the maintenance of cells which are slightly more mature.

The large cells of Figs. 6 and 8 are from marrow smears of a patient with macrocytic anemia and normoblastic hyperplasia showing marked shift to the left in normopoiesis. Final diagnosis had not been established at the time of this study. Large primitive erythroblasts similar to those of Figs. 6 and 8 were quite numerous. Many of the older developmental stages were abnormally large but were clearly members of the normoblastic series. Normoblasts of normal size were also present. The erythrocytes were of corresponding difference in size, from which we assume that the macrocytes were developed from the larger normoblasts. Because of the numerous large primitive erythroblasts and the macrocytes, it was at first thought that this might be a case of megaloblastic anemia. However, careful search through several marrow slides failed

to reveal any erythroblasts that had developed beyond the stage illustrated in Fig. 6 as megaloblasts. Although many were large, all were definitely normoblasts.

Comparison of cell 6 with the other cells of Plate I, which are from specimens taken from patients having pernicious anemia, shows that the cell possesses features that are usually thought to be characteristic of promegaloblasts, or the "erythrogones" or "proerythroblasts" of some authors. It has the stippled nucleus with no clumping of chromatin, three irregular-shaped nucleoli, a thin nuclear membrane, and abundant cytoplasm that is similar to that of many promegaloblasts or erythrogones. We are certain that if one confined his examination to cells of this type and disregarded the numerous normoblasts of this marrow he would conclude that these were primitive erythroblasts which would develop to megaloblasts. However, in this case they belong clearly to the normoblastic series. The large cell of figure 8 from the same slide is a slightly older stage in the development toward normoblasts. It still has the stippled nuclear pattern and three irregular nucleoli. However, there is some clumping of chromatin, and the cytoplasm is less abundant and has a wider and more basophilic rim. In spite of the general chromatin pattern and the nucleoli we would place cells of this type with the normoblasts.

Large "proerythroblasts," identical with those of our Figs. 1 to 6, Plate I, are shown in Fig. 30 of the monograph *La Talassemia* by Astaldi, Tolentino and Sacchetti. Although these cells have been interpreted as megaloblasts, the authors found that the successive differential stages of these cells are normoblasts. They considered the cells to be "intermediate stages of direct erythroblastic evolution of histioid cells." They did not find any megaloblasts in the marrow in cases of thalassemia.

On account of the narrow and relatively homogeneous rim of cytoplasm one might be inclined to classify the left-hand erythroblast of Fig. 4 as a very immature pronormoblast. However, in this case one can easily trace the intermediate forms from this type of cell to those which are definitely megaloblastic. This patient was in extreme relapse and had not been treated. Normoblasts were very scarce; we searched the marrow film for some time before finding one. There were a few small orthochromatic cells with pyknotic nuclei which could be normoblasts but, with few exceptions, those with an open nucleus were definitely megaloblastic. Cells like the one of figure 1 were fairly numerous in this marrow, and there is good evidence for a reticular origin of the most primitive erythroblasts of this case. As the cells mature their cytoplasm becomes strongly basophilic before there is any change from the reticular type of nucleus.

From what we have said and illustrated, it is evident that we have encountered the same difficulty in classifying the earliest erythroblasts that others have had with this problem. Certain trends can be recognized, but individual primitive erythroblasts cannot always be characterized as either normoblastic or megaloblastic. This, of course, is true of only the most immature types of erythroblasts which appear with extreme shift to the left in erythropoiesis, as in pernicious anemia in relapse and in certain cases of extreme normoblastic

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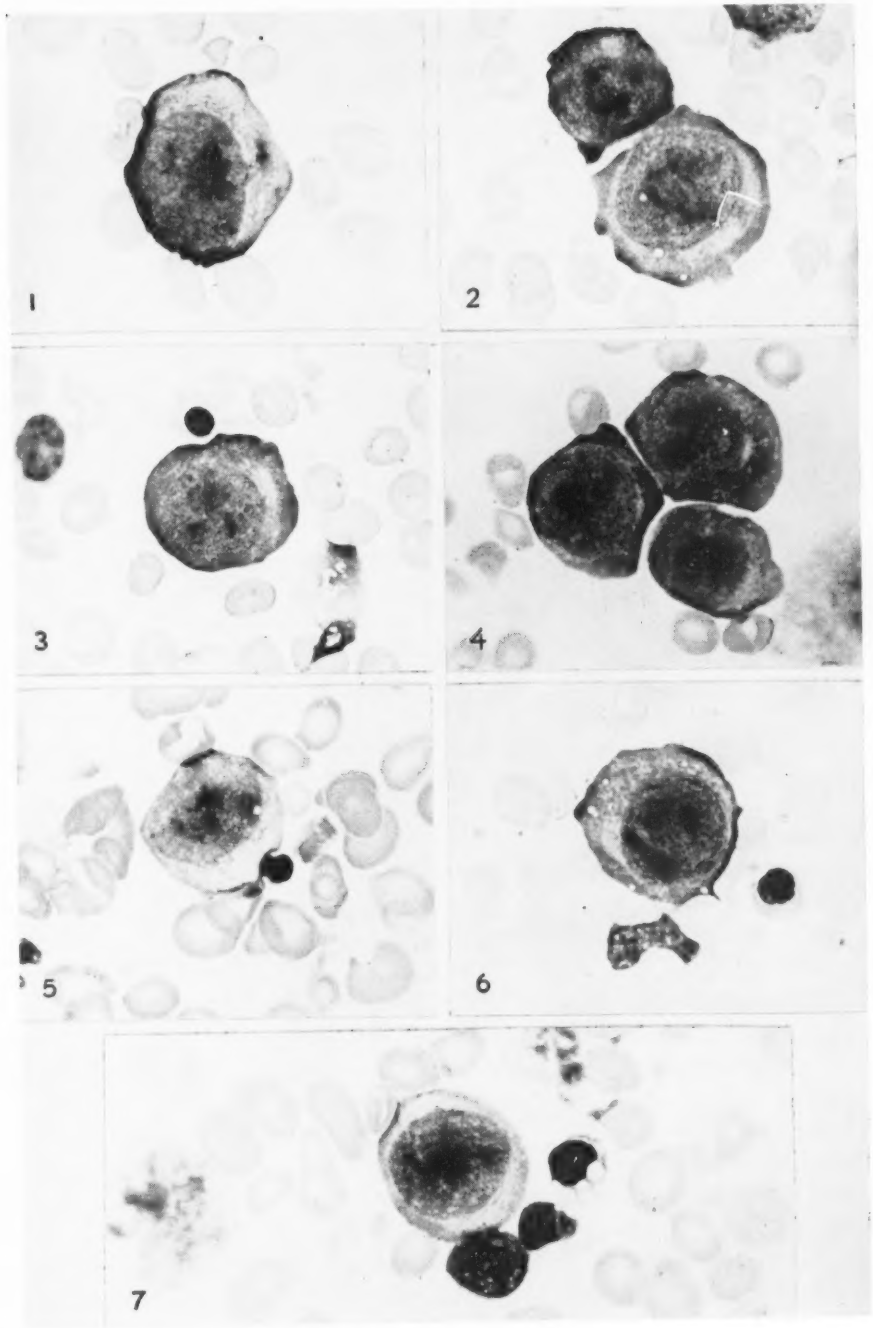

Explanation of Figures.—

All figures are photographs, made by the Section of Photography of the Mayo Clinic, of cells from aspirated sternal bone marrows of living patients. They were photographed at a magnification of 800, except Figs. 16 and 17, which were photographed at a magnification of 1,200. All are reproduced without reduction. Staining was with Wright's stain.

Plate I.—

Figs. 1 to 4.—Examples of the most immature erythroblasts ("erythrogones") in untreated pernicious anemia in relapse. Fig. 1 is the most immature cell of the group. It probably is an intermediate stage between a free reticular cell and a primitive erythroblast. Note variations in cell size and degree of basophilia, and the irregular-shaped and faintly outlined nucleoli. Figs. 1 to 3 are from the same slide. Fig. 4 is from a different patient.

Fig. 5.—Very primitive erythroblast, or cell that is intermediate between reticular cell and erythroblast. Compare with Fig. 1. From case of pernicious anemia forty-eight hours after treatment with folic acid began. From slide donated by E. M. Schleicher and treated with his decolorizer.

Fig. 6.—Primitive erythroblast from case of macrocytic anemia, normoblastic hyperplasia, and marked shift to left in normopoiesis. Note similarity to the "erythrogones" of Figs. 2, 3, and 4.

Fig. 7.—Promegaloblast and normoblasts. Same slide as that from which Fig. 5 was made.

hyperplasia. The morphologic characters that determine the classification of the products of differentiation have not been impressed on these most primitive types of erythroblasts. Those that will eventually develop to megaloblasts tend to be larger and more irregular in shape and have a wider rim of cytoplasm which is less homogeneous than is true of the corresponding cells which will develop to normoblasts. However, if individual cells are studied it will be noted that there is great variation in these characters, no one or combination of which predetermines the direction of differentiation.

In untreated pernicious anemia in relapse we have noted great variations from case to case in the character of the most primitive erythroblasts and in the promegaloblasts. In some cases the very large cells, like those of Figs. 1, 2 and 3, are numerous, while in others they are scarce, most of the young cells being like the smaller ones of Figs. 2 and 4. To some extent these differences depend on the degree of relapse as estimated on the basis of the hemoglobin percentage and the erythrocyte count, but there are exceptions to this.

Before treatment the marrow of this patient contained many large primitive erythroblasts, examples of which are shown in Figs. 1, 2, and 3. Besides these large cells this marrow contained a goodly number of smaller erythroblasts, like the upper one of Fig. 2 and the smallest one of Fig. 4, in which the nuclear pattern is just as fine and the irregular nucleoli just as prominent as in the larger cells. Twelve hours after the intramuscular injection of 15 units of Lederle's liver extract the number of these smaller cells had increased, while the number of the larger cells had diminished. There was a conspicuous increase in the number of mitoses. Most of the nuclei of these cells were similar to those in the corresponding cells before treatment, but some had differentiated to a slight extent and resembled the nuclei of small promegaloblasts. There were no normoblasts at this stage. This marrow picture at twelve hours could be interpreted as the first change toward the normoblastic marrow but, from the morphologic pattern of the cells, it could also be interpreted as an indication of rapid megaloblastic maturation. An additional marrow aspiration was not done. However, since the patient showed the typical response to treatment, we must assume that in this case the development of numerous small basophilic primitive erythroblasts is the earliest change toward a normoblastic marrow.

Because the small erythroblasts were fairly numerous before treatment it might be assumed that this patient was not in complete relapse. Pernicious anemia had been diagnosed elsewhere in 1944. Treatment was continued for one year. Symptoms recurred in 1946. The patient was admitted to the clinic in May, 1947, with a red cell count of 1,530,000 per cubic millimeter of blood and hemoglobin of 7.7 Gm. per 100 ml. of blood. He received 15 units of liver extract daily for two days and 15 units on the seventh day. Reticulocytes were 12.8 per cent on the eighth day.

Small promegaloblasts and basophilic megaloblasts can be numerous when the erythrocyte count is quite low. Another patient was admitted with an erythrocyte count of 1,600,000 per cubic millimeter of blood and reticulocytes of 0.3 per cent. Before treatment his marrow contained few large promegaloblasts but many of the smaller promegaloblasts and basophilic megaloblasts. He

showed good response to daily injections of 100 mg. of folic acid. Polychromatic normoblasts were very numerous on the third day after treatment began. Reticulocytes were 10.2 per cent on the sixth day. Erythrocytes were 4,120,000 per cubic millimeter of blood three months after treatment began.

Two other patients having pernicious anemia had few large promegaloblasts or primitive erythroblasts in their marrow before treatment but many of the smaller forms. In one the erythrocyte count was 1,730,000 per cubic millimeter of blood and hemoglobin 8.8 Gm. per 100 ml. of blood. The other patient had an initial count of 1,960,000 per cubic millimeter of blood and hemoglobin 6.0 Gm. per 100 ml. of blood.

We conclude from these observations that it is not always possible to determine the direction of differentiation from the morphologic characters of the most primitive erythroblasts. Their size is extremely variable from case to case and in the marrow of the same case. Some marrows, before treatment, are characterized by the great number of large primitive erythroblasts and promegaloblasts, while in other marrows these cells are much smaller although the degree of maturation is no greater. With normoblastic hyperplasia the most primitive erythroblasts tend to be large, as in our Fig. 6, but there is rapid reduction in size as the cells divide and mature. In some of our cases of untreated pernicious anemia there are many late megaloblasts which are much larger than the corresponding late normoblasts, except in some macrocytic anemias and in some conditions in which the normoblasts are involved in some malignant process. However, since the late polychromatophilic megaloblasts can be as small as the normoblasts of equal maturity, size is not a reliable criterion for classification of erythroblasts at any stage of their development.

We agree that megaloblasts and normoblasts constitute two separate series of erythrocytes which can usually be distinguished without difficulty after they have reached the "pro" or later stage of their development. However, these two lines converge on a primitive erythroblast which has no distinguishing megaloblastic or normoblastic features. If one or more of the anti-megaloblastic anemia factors are lacking, this "erythrogone" or "proerythroblast" will undergo pathologic development to a megaloblast. If these factors are present or supplied it will develop to a normoblast.

In the abnormal megaloblastic series pathologic features, such as atypical mitoses, displaced chromosomes, excessive karyorrhexis, vacuoles in cytoplasm, and so forth, usually do not appear until some hemoglobin has developed, and it is at this stage that the cells are affected by arsenic in the form of Fowler's solution, as described by Rohr[53] and by Limarzi.[42] The essential changes resulting from the arsenic treatment are precocious development of hemoglobin and excessive karyorrhexis. Since Limarzi has shown that the proerythroblasts and the promegaloblasts are not affected by the arsenic, it can be assumed that these cells have not been affected by the lack of the antianemic factor to the extent that they cannot develop to normoblasts if the missing factor is supplied. Limarzi found that the older normoblasts in hereditary hemolytic jaundice show about the same pathologic features after administration of arsenic as are

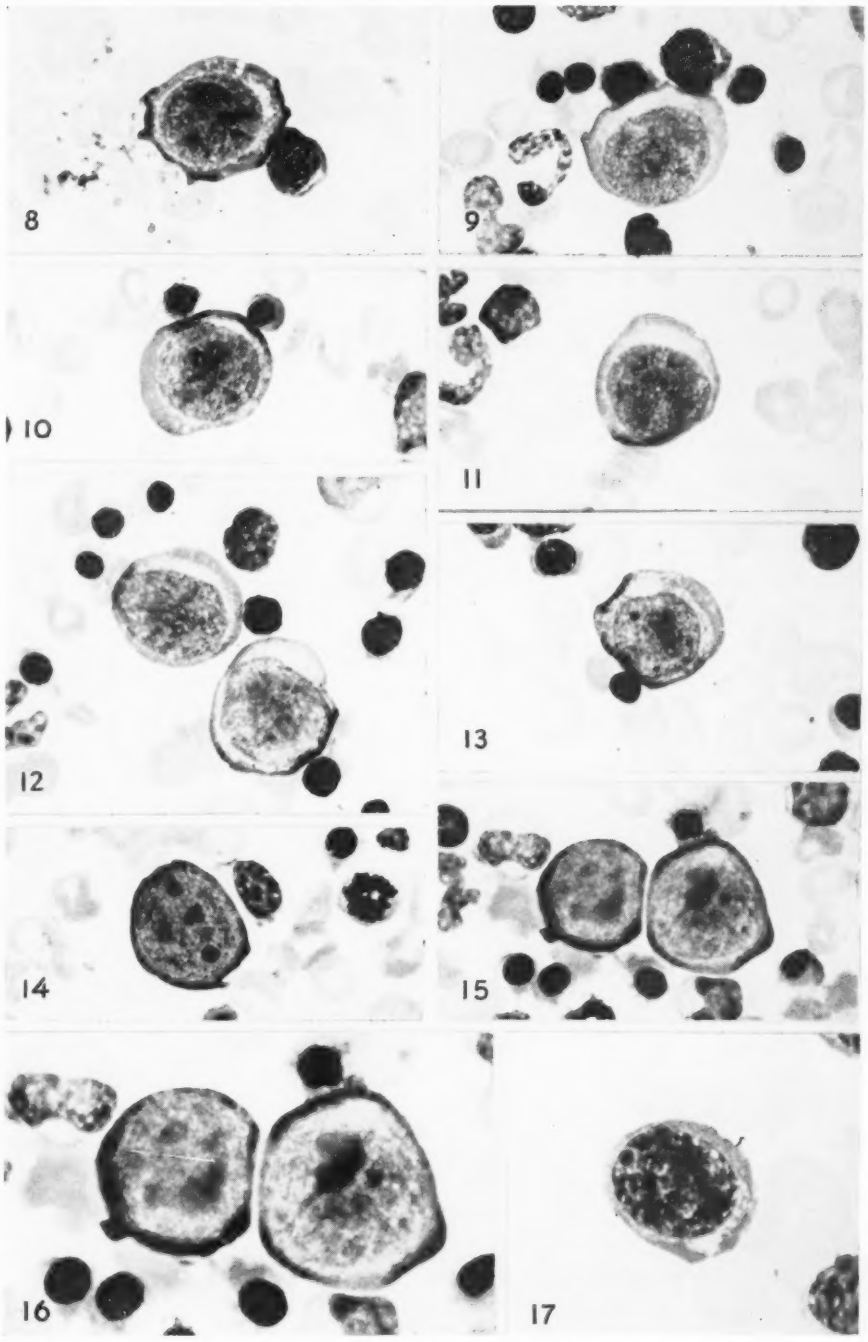

Plate II.—

Fig. 8.—Early pronormoblast. Same slide as Fig. 6.

Figs. 9 to 12.—Erythroblasts from hemolytic anemia. Figs. 9, 11, and 12 have nuclei that are quite similar to those of the primitive erythroblasts of pernicious anemia. Fig. 10 is an early pronormoblast. Figs. 9, 11, and 12 are from slides donated by E. M. Schleicher. Fig. 10 is from a slide donated by Dr. Dorothy Sundberg.

Fig. 13.—Primitive erythroblast from case of treated megaloblastic anemia following intestinal operation. Free hydrochloric acid. Good response to folic acid.

Figs. 14 and 15.—Basophilic pronormoblasts, hemolytic anemia. Note clumping of chromatin and large irregular nucleoli in the two large cells of Fig. 15. Sharply outlined nucleoli and stippled chromatin pattern in large cell of Fig. 14.

Fig. 16.—Same as Fig. 15 but at a magnification ×1,200.

Fig. 17.—Polychromatophilic normoblast from case of treated pernicious anemia. Typical normoblastic nuclear pattern. (Magnification ×1,200.)

seen in the later megaloblasts, and that the pronormoblasts and basophilic normoblasts show no such alterations. Usually normoblasts at any stage of their development do not show these changes after administration of arsenic.

We have shown that the erythrogones of pernicious anemia vary in size, shape and number of nucleoli, and in basophilia of cytoplasm as expressed by the amount and distribution of the spongioplasm, and that all have a very primitive, more or less reticular type of nucleus with uniform distribution of chromatin in the form of small irregular-shaped granules. Jones (1938)[31] has given a detailed description of this nucleus for the youngest erythroblasts which are developing from reticulum. We find similar nuclei in proerythroblasts that will develop to normoblasts, as shown in our Fig. 6. This nucleus can be distinguished from that of the myeloblast without difficulty. In the latter the chromatin is usually arranged in the form of a very delicate network, so that this nucleus does not have the stippled appearance seen in the earliest proerythroblasts. The nuclei of both series may have a fine chromatin network when the proerythroblasts have developed to some extent. This is particularly true of the normoblasts. In the megaloblastic series the stippling tends to become coarser and a network does not appear until later. For these reasons Ferrata, Storti,[64, 65] and others we have mentioned, derive the megaloblasts from free cells of the reticulum and normoblasts from hemocytoblasts (myeloblasts). In Jones's opinion the myeloblast is the usual progenitor for both series, and both may be derived from reticulum under certain conditions without passing through the myeloblast stage, as illustrated in his colored plate (1938).

Some myeloblasts are also derived from reticulum, a fact which would account for the occasional myeloblast with a finely stippled chromatin pattern in place of the usual sievelike chromatin network. These two types of myeloblasts were described and illustrated by Downey in 1927.

In the marrows of our cases of untreated pernicious anemia myeloblasts are very scarce, and we do not find much evidence for the derivation of proerythroblasts from them. They are more numerous one or two days after the beginning of treatment. They are fairly numerous in our cases of normoblastic hyperplasia, and we have seen some pronormoblasts that seem to have been derived from myeloblasts. However, we could not get any evidence in favor of the derivation of cells like the one in Fig. 6 from myeloblasts.

On Plate II we illustrate primitive erythroblasts from the marrow in cases of hemolytic anemia (Figs. 9 to 12 and 14 to 16) and from a case of treated megaloblastic anemia which developed after an intestinal operation (Fig. 13). The marrow films from which Figs. 9, 11, and 12 were made were donated by Mr. E. M. Schleicher and were treated with his decolorizer. Fig. 10 is from a slide donated by Dr. Dorothy Sundberg. The decolorizer removes the dye that is adsorbed on the surface of the chromatin strands, resulting in a sharper definition of the nuclear pattern. However, equally good results can be obtained without the use of the decolorizer, as is shown by the sharp nuclear pattern in Fig. 13. Cells that have been decolorized usually are not quite as basophilic as those that have not been so treated.

There were no megaloblasts in the marrows from which the cells of Plate II were photographed, but the chromatin pattern and general appearance of several of these cells closely resemble those of the primitive erythroblasts of the megaloblastic marrows, as shown in Figs. 9, 11, 12, and 13. Nucleoli are irregular in shape and usually faintly stained. However, they may be well stained and sharply outlined even in the pronormoblast or basophilic normoblast stage, as shown in Figs. 14 and 15.

Cell 9 has the typical "erythrogone" nuclear pattern except that the pattern is interrupted in two places, which might be due to damage. In Figs. 11 and 12 the nucleoli are large and faintly outlined and are difficult to separate from the chromatin pattern. The two cells in Fig. 12 are rather typical erythrogones similar to many in pernicious anemia marrow. The lower cell in this figure resembles cell 5, from a case of pernicious anemia. The cytoplasm of the upper cell has developed a wide homogeneous basophilic rim beyond the juxtanuclear area, which might be interpreted as beginning maturation of the cytoplasm. However, the nucleus of this cell shows no signs of beginning maturation; there is no clumping or radial arrangement of chromatin; the wide radiating streaks are composed of nucleolar material. In Fig. 10 the condensation of cytoplasm has progressed farther and the nucleus shows beginning clumping of chromatin. In spite of the scattering of nucleolar material we interpret this cell as an early pronormoblast.

The general appearance of the cells in Figs. 14 and 15, from cases of hemolytic anemia, places them among the basophilic normoblasts. They have the characteristic narrow basophilic cytoplasm, but their nuclei are still quite undifferentiated and they have prominent nucleoli. They are the "proerythroblasts" of Naegeli, but not the proerythroblasts of Schulten, Israëls, and Schartum-Hansen,[56, 57] which are the more primitive erythrogones of other authors. Cell 14 is unique because of its several sharply outlined nucleoli and stippled chromatin pattern like that of the erythrogones of Plate I. This stippled appearance is produced by the light, practically unstained, rounded, evenly spaced "parachromatin," which seems to be embedded in the dark chromatin in the form of unstained granules. The details of this nuclear pattern are clearly seen if these photographs are examined with a reading glass. It is a pattern that is characteristic of the nuclei of primitive erythroblasts and of many nuclei of endothelial and reticular cells. Myeloblasts have a somewhat similar chromatin pattern, but it is much finer in texture with much thinner chromatin strands which usually do not stain as deeply as do those of the erythroblast.

The basophilic normoblasts of Fig. 15 have large but not sharply outlined nucleoli, and the chromatin shows definite clumping. We have tried to emphasize this by photographing these same cells at a magnification of 1,200, as seen in Fig. 16. As these basophilic normoblasts divide and mature, the chromatin clumps become more clearly defined and denser, and there is sharper separation of chromatin from parachromatin until the polychromatophilic stage of Fig. 17 is reached. At this stage the parachromatin is often oxyphilic in

staining reaction, and nucleoli have disappeared. The cell of Fig. 17 was also photographed at a magnification of 1,200 in order to emphasize the nuclear pattern.

As the megaloblasts mature, the open nuclear pattern of the promegaloblast of Fig. 7 is maintained for some time, but the chromatin strands become thicker and the interspaces wider. This is followed by the formation of irregular clumps of chromatin which are more widely separated than those of the normoblasts of the same stage. The parachromatin is, therefore, quite conspicuous. The chromatin blocks are usually connected by fine chromatin strands which tend to be longer than those of the denser nucleus of the normoblast. At this polychromatophilic stage there is no difficulty in distinguishing between megaloblast and normoblast. These later stages should not be ignored, as is done by several authors we have mentioned. They facilitate identification of a marrow as megaloblastic or normoblastic when it is difficult to classify the earliest stages.

The erythroblast of Fig. 13 is of interest because of its lacy, wide-meshed chromatin network, the four dark chromatin granules, and its indefinite nucleolus. The marrow from which this cell was photographed was normoblastic and macronormoblastic, and careful search did not reveal any cells that could be classified as megaloblasts. However, this patient did have megaloblastic anemia which showed good response to folic acid therapy. Cell 13 is from marrow taken seven days after daily injection of 20 mg. of folic acid. This marrow contains some "erythrogones" similar to those of untreated pernicious anemia, and a few intermediate cells. Before treatment it contained many erythrogones and promegaloblasts, and megaloblasts and intermediate cells in various stages of development. There were very few typical normoblasts. The neutrophilic leukocytes and their promyelocytes and myelocytes were of the pernicious anemia type (see Jones, 1938[31]). Cells similar to the one of Fig. 13 were also found in our untreated pernicious anemia marrows, where they were related to the megaloblastic series. However, in the pernicious anemia marrows the dark spherical nuclear granules were composed of nucleolar material, as indicated by the staining reaction.

A brief résumé of the history of the patient from whom the cell of Fig. 13 was taken may be of interest. In May, 1944, he underwent right hemicolectomy and ileocolostomy for an obstructive lesion of the ileum. He returned to the clinic two years later (April, 1946) with moderately severe macrocytic anemia. His hemoglobin was 7.1 Gm. per 100 ml. of blood, erythrocytes 1,790,000, and leukocytes 7,100 per cubic millimeter of blood. Color index was 1.31 and erythrocytometer reading 8.2 microns. Free hydrochloric acid was present in the gastric juice. Persistent diarrhea had been present since the operation in 1944. Stools contained 63 per cent of fat. Bone marrow taken at this time showed the features described previously for the pretreatment marrow.

Daily parenteral administration of 20 mg. of folic acid was begun May 3, 1946. The diarrhea became less severe within a week. A reticulocyte peak of 18.1 per cent was reached on the eighth day, and the erythrocyte count rose steadily from the pretreatment level of 1,790,000 per cubic millimeter of blood to 3,900,000 on June 12. No other type of therapy was given. Diarrhea had practically disappeared when the patient was dismissed on June 19, 1946. He had gained weight and felt quite well. At this time oral folic acid therapy, 30 mg. daily, was begun, and this has been continued to date. Two months later hemoglobin was

12.3 Gm. per 100 c.c. of blood, erythrocytes 4,270,000 per cubic millimeter of blood and color index 0.95. In June, 1948, the figures were hemoglobin 13.8 Gm. per 100 c.c. of blood, erythrocytes 4,320,000, and leukocytes 7,700 per cubic millimeter of blood, and color index 1.08.

On April 19, 1950, the patient wrote that he was feeling very well and was working. He has continued the folic acid therapy. He stated that his blood counts remained normal, but he did not give us the evidence for this.

We might assume that the megaloblastic anemia was due to folic acid deficiency resulting from the intestinal operation. However, liver or vitamin B_{12} therapy was not tried. The cytologic transformation of the marrow, seven days after the beginning of treatment in May, 1946, was not as complete as expected in pernicious anemia after similar therapy. Megaloblasts had disappeared, but a few primitive erythroblasts (erythrogones) and intermediate cells were still present, and basophilic normoblasts were more numerous than they would be in a case of pernicious anemia seven days after the beginning of treatment.

Cell 13 was photographed because it shows one type of structural variation which may be encountered among the primitive erythroblasts. Similar cells are scarce but were seen in marrow in several cases of untreated pernicious anemia.

B. Normoblasts in Bone Marrow of Untreated Pernicious Anemia Patients in Relapse.—Normoblasts and cells that could be classified as intermediate erythroblasts were scarce in many of our marrows taken before treatment and often could be found only after careful search. As examples we have a group of four patients with hemoglobin of 6 to 8.8 Gm. per 100 ml. of blood and erythrocytes 1,530,000 to 1,960,000 per cubic millimeter of blood. Myeloblasts were also very scarce in the pretreatment marrows of this group of patients.

Normoblasts were present in the pretreatment marrows of another group of four patients who had hemoglobin percentages and red cell counts that did not differ greatly from the corresponding figures of the previous group. The lowest hemoglobin in the group was 6.3 Gm. per 100 ml. of blood in a patient who had an erythrocyte count of 1,630,000 per cubic millimeter of blood. The highest hemoglobin in the group was 10 Gm. per 100 ml. of blood in a patient who had an erythrocyte count of 1,640,000 per cubic millimeter of blood. The highest erythrocyte count in this group was 1,870,000 per cubic millimeter of blood (hemoglobin 7.3 Gm. per 100 ml. of blood), the lowest 1,630,000 per cubic millimeter of blood (hemoglobin 6.3 Gm. per 100 ml. of blood). The patient who had the highest hemoglobin would have been placed in the next group of six patients if it had not been for his low erythrocyte count. His marrow contained a fair number of promegaloblasts and basophilic megaloblasts, but erythropoiesis was primarily normoblastic. The reticulocytes rose from 0.6 per cent to 7.0 per cent after daily treatment with 1 unit of liver extract parenterally and 1 mg. of folic acid orally.

In the next group of six patients normoblasts and cells classified as intermediate erythroblasts were numerous, and in two of these patients they were the only types of erythroblasts present. The lowest hemoglobin of this group was 9.0 Gm. per 100 ml. of blood, and the lowest erythrocyte count was 2,150,-

000 per cubic millimeter of blood in the same case. The highest hemoglobin was 11.7 Gm. per 100 ml. of blood, and the highest erythrocyte count was 2,920,000 per cubic millimeter of blood in the same case.

The absence of megaloblasts from the marrow of one of the two patients without megaloblasts may be due to an intramuscular injection of liver extract received one week before he came to the clinic. At the clinic his initial hemoglobin was 10.1 Gm. per 100 ml. of blood and erythrocyte count 2,430,000 per cubic millimeter of blood. The diagnosis was pernicious anemia in relapse. There was slow response to oral administration of folic acid, 100 mg. daily. Reticulocytes were 6.4 per cent on the fourteenth day.

The other patient without megaloblasts, had an initial hemoglobin of 11.7 Gm. per 100 ml. of blood and 2,920,000 erythrocytes per cubic millimeter of blood. The blood picture was negative for pernicious anemia except for an occasional neutrophilic leukocyte of the pernicious anemia type. The erythroblastic part of the marrow picture was composed of macronormoblasts, normoblasts, intermediate erythroblasts, and a few erythrogones. A few of the neutrophilic promyelocytes and myelocytes were of the type characteristic of pernicious anemia. There was also slow response to daily intramuscular injections of 1 microgram of vitamin B_{12} concentrate. Reticulocytes were 4.2 per cent on the seventh day. Diagnosis would have been uncertain if study of the patient had been confined to the initial blood and marrow pictures.

The third of these three groups of patients has been described in order to show that in untreated patients with hemoglobin levels between 9 and 12 Gm. per 100 ml. of blood and erythrocytes between 2,000,000 and 3,000,000 per cubic millimeter of blood one may expect a mixed megaloblastic-normoblastic and intermediate cell picture, or erythropoiesis will be normoblastic and macronormoblastic, with some erythroblasts of the intermediate cell type. One of the two patients with normoblastic erythropoiesis had no previous specific therapy. However, we could see no important differences in the marrows from the two patients.

The first two groups show that, with hemoglobin levels of from 6 to 10 Gm. per 100 ml. of blood and erythrocytes between 1,000,000 and 2,000,000 per cubic millimeter of blood, normoblasts in any considerable number and intermediate erythroblasts may or may not be present. Some authors (Chevallier,[6] Bilski-Pasquier, and others) have claimed that normoblasts are always present in the pretreatment marrows, even when the patients are in extreme relapse. We do not dispute this claim. However, normoblasts are so scarce in some marrows that prolonged searching through the preparations is required to locate an occasional one.

C. Bone Marrow Alteration Following Specific Therapy in Pernicious Anemia.—Opinions concerning the early changes which occur in bone marrow of pernicious anemia patients following specific therapy were mentioned in our review of the literature. The general changes in the marrow picture following adequate treatment are well known and need not be discussed here. Differences of opinion concern some of the details which are difficult to follow in the rapid transformation resulting from the customary therapy.

It has been shown, especially by Rohr[53, 54] and Schartum-Hansen,[56, 57] that small doses of the specific therapeutic agent used will cause marrow transformation of the same type as that which follows adequate treatment, but the change to normoblastic marrow will be slow and often incomplete. For this reason we

felt that some of the disputed details could best be studied with small dosage, and that the information would be more reliable than that obtained from a study of marrow of untreated patients in partial relapse, or from relapsed patients after the customary dosage. However, several of our patients who had received adequate treatment were favorable for study of the early marrow changes. In three of these who were studied in detail the earliest marrow aspiration was done twelve hours and in one of them twenty-four hours after the beginning of treatment.

Two patients received daily intramuscular injections of very small amounts of Lederle's liver extract after the pretreatment marrow had been studied. One received ½ unit daily, and aspirations were done twenty-one and forty-eight hours later. The other patient received 1 unit daily, and aspirations were done at twenty-four and forty-eight hours, and six days.

Four patients were treated with small amounts of both liver extract and folic acid. The liver extract was administered parenterally in all four cases. One of these patients received 5 units of liver extract parenterally and 5 mg. of folic acid by mouth. A second patient received 5 units of liver extract parenterally and 5 mg. of folic acid parenterally. In the third, the dosage was 5 units of liver extract parenterally and 2 mg. of folic acid by mouth, and in the fourth ½ unit of liver extract parenterally and 5 mg. of folic acid by mouth. Sternal aspirations were at twenty-four hours for the first patient, six and thirty-six hours for the second, forty-eight hours and five days for the third, and twenty-six and forty-five hours for the fourth.

The observations recorded in the following pages are based chiefly on detailed study of marrows of the foregoing patients, since they proved to be the most favorable ones of the forty-five patients examined.

D. The Megaloblasts During Treatment.—The small "erythrogones" and promegaloblasts, such as the smaller cells of Figs. 2 and 4, were discussed earlier in this paper. They are always present in the pretreatment marrows of relapsed patients, but their relative number compared to the larger cells, like those of Figs. 1, 2, and 3, varies from case to case. As a rule they are more numerous with the higher levels of hemoglobin and erythrocyte counts, but there are exceptions to this.

Our material does not answer the question whether the presence of a greater number of these smaller cells signifies that the patient is not in the same degree of relapse as the one who has fewer small and more large primitive erythroblasts in his marrow. That this is so might be assumed from the great increase of the smaller cells in twelve to twenty-four hours after the beginning of treatment. The larger cells are greatly reduced in number and in some instances have almost disappeared at twenty-four hours. In four of our cases this change occurred before the appearance of normoblasts. In the early hours the small cells might show little evidence of beginning maturation, especially with inadequate medication, but mitoses were numerous. It may be assumed, therefore, that one of the first changes after the beginning of treatment is the reduction by mitosis in the size of the larger erythrogones and promegaloblasts.

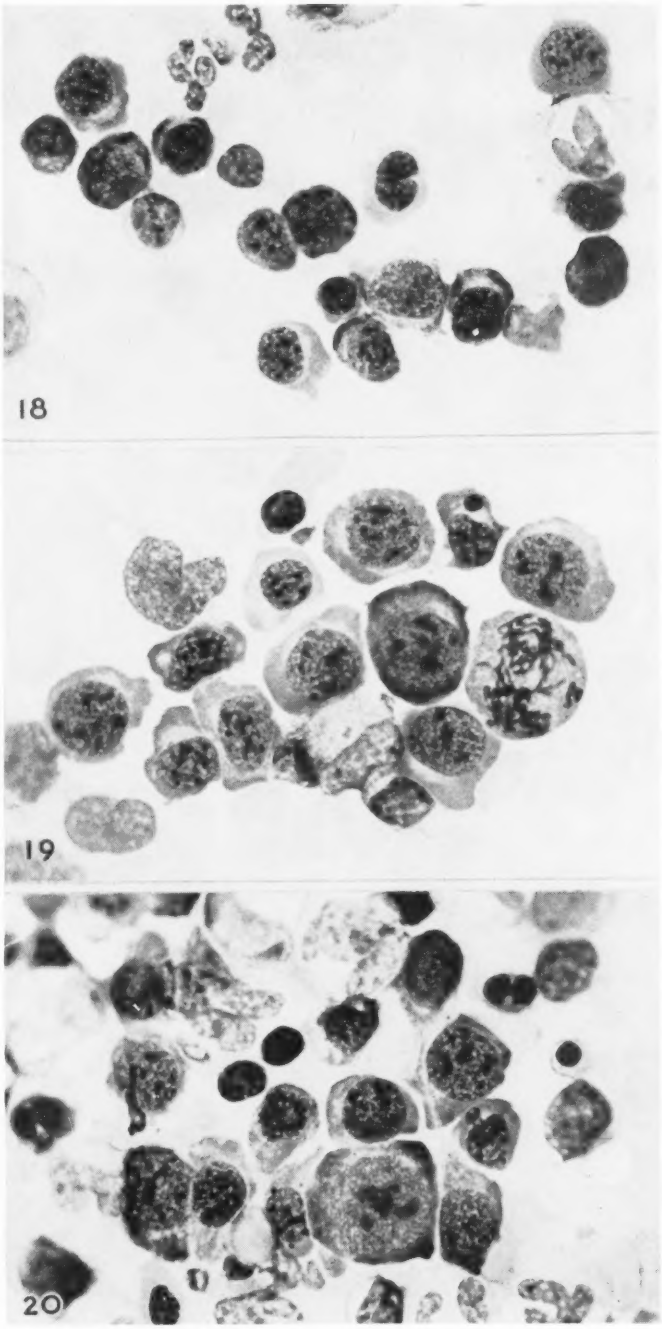

Plate III.—

Fig. 18. "Intermediate erythroblasts," pernicious anemia, twenty hours after injection of 15 U.S.P. units of liver extract. The small size of these cells probably is due to the rapid transformation of this marrow. Some megaloblasts are still present, but most of the erythroblasts are of intermediate structure. Some of the nuclei are of early normoblastic structure. (Magnification ×800.)

Figs. 19 and 20.—"Intermediate erythroblasts," pernicious anemia, forty-eight hours after first daily injection of 1 U.S.P. unit of Lederle's liver extract. The two figures are from the same marrow film. Since transformation of this marrow was much slower than transformation of that from which Fig. 18 was made, the early transitional stages from the large "erythrogone" to the intermediate cells of each figure are larger and more numerous than in the rapidly transforming marrow from which Fig. 18 was made. Note atypical mitoses in Figs. 19 and 23. (Magnification ×800.)

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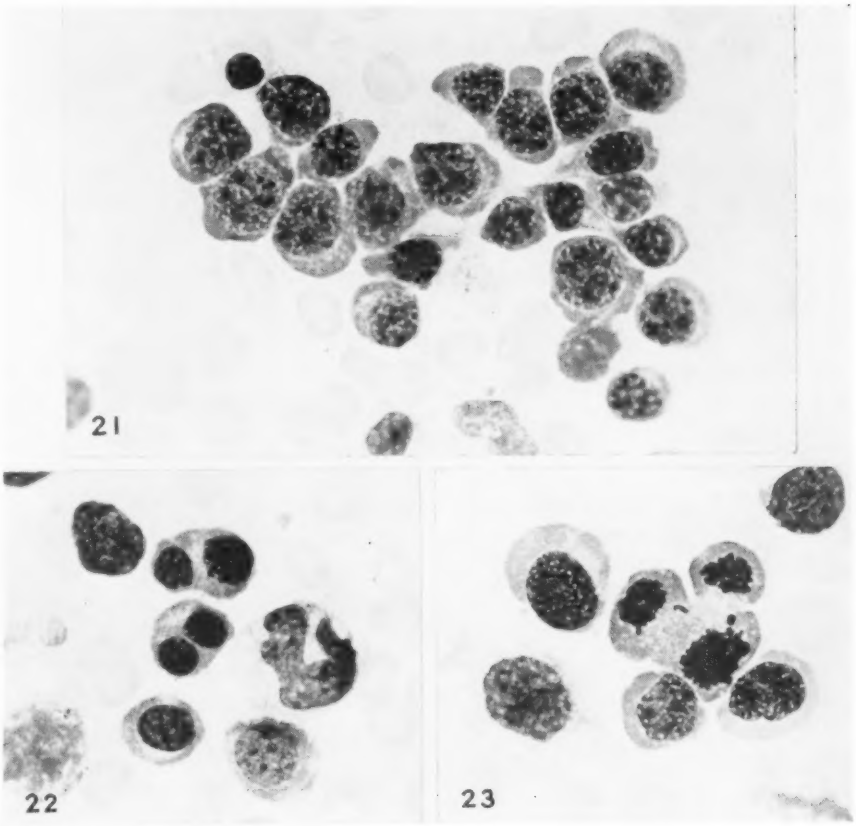

Plate IV.—

Fig. 21.—Pernicious anemia. Normoblastic transformation forty-eight hours after first daily injection of $\frac{1}{2}$ U.S.P. unit of liver extract. The pretreatment marrow did not show the picture of complete relapse, a fact which probably explains the excellent response to this small dosage. Cytoplasm of all the cells shows the same degree of polychromasia, but nuclei are in different stages of normoblastic transformation. A few of the nuclei of the larger cells are still in the early intermediate stage. Note nucleoli in some of the cells.

Figs. 22 and 23.—Pernicious anemia, twenty-four hours after administration of 5 mg. of folic acid by mouth and 5 U.S.P. units of liver extract parenterally. Intermediate erythroblasts, two polychromatophilic normoblasts with double nuclei, and one large pernicious anemia type leukoblast in Fig. 22. Fig. 23 shows intermediate erythroblasts and an atypical triple mitosis with isolated chromosomes.

This rapid increase in the number of small cells has been noted by others, especially Schleicher (1945),[59] who referred to them as "normoblast-like erythroblasts" which will not mature to true normoblasts.

In some cases not all the larger primitive erythroblasts disappear until later, a few being present at thirty-six and forty-eight hours. If the small cells are numerous before treatment they are greatly increased in the early hours after treatment. This is also true of the normoblasts. For this reason the marrow changes resulting from treatment with minimal effective doses are seen to best advantage in those cases in which the pretreatment marrow contains few normoblasts. Soon after the beginning of treatment any young normoblasts present will proliferate rapidly, while at the same time most of the larger erythrogones and promegaloblasts also multiply, are reduced in size, and gradually transform to the "intermediate erythroblasts." The speed of these changes depends on the adequacy of the medication and the type of marrow which existed before treatment was begun.

A few large erythrogones and promegaloblasts still may be present in the twenty-four hour and forty-eight hour marrows along with late megaloblasts. We could not decide whether the former had persisted from the pretreatment marrow or whether they were newly formed. A few orthochromatic megaloblasts were found in four-day and six-day marrows of patients who were receiving suboptimal treatment. One of the four-day marrows contained some erythrogones and numerous pronormoblasts but no young megaloblasts. The erythrogones were identical with those of the pretreatment marrow but clearly were related to the pronormoblasts in the four-day marrow.

In the opinion of Jones,[31, 32, 33] Fieschi,[19, 20] Storti,[64] and Scott most of the megaloblasts complete their maturation. Lambin and de Weerdt,[38] and Henning and Keilhack found that only a few mature, and Schartum-Hansen[57] stated that they all degenerate before reaching the megalocyte stage, and that "megalocytes" and macrocytes of the blood are derived from "macroblasts" (pronormoblasts).

We find great differences in maturation activity of megaloblasts in the pretreatment marrows of our cases. In some marrows erythrogones and the younger megaloblasts, including the early polychromatophilic stages, were numerous, while the older stages were scarce. In other patients maturation was active and all stages were present and numerous. In two of our patients all developmental stages were so numerous that one could hardly speak of "maturation arrest" in these marrows.

The first patient had an initial hemoglobin reading of 6.2 Gm. per 100 ml. of blood and erythrocytes 1,680,000 per cubic millimeter of blood. She was treated daily with 2 mg. of folic acid by mouth and 1 U.S.P. unit of Lederle's liver extract parenterally. Reticulocytes were 14 per cent on the sixth day. Marrow taken forty-eight hours after the beginning of treatment contained many basophilic and polychromatophilic normoblasts, a few polychromatophilic megaloblasts, and many intermediate erythroblasts. Late megaloblasts were persent but not numerous. The five-day marrow was normoblastic with a few large pronormoblasts. Three late megaloblasts were seen.

The second of the two patients with active megaloblastic regeneration had an initial hemoglobin reading of 6 Gm. per 100 ml. of blood and erythrocytes 1,960,000 per cubic milli-

meter of blood. He was given daily injections of 1 U.S.P. unit of Lederle's liver extract. Reticulocytes were 20.6 per cent on the eighth day. This patient had had carcinoma of the rectum which had been resected in 1946. He returned to the clinic in 1947, and a diagnosis of pernicious anemia in relapse was made. His pretreatment marrow contained many large erythrogones and promegaloblasts, also many medium-sized and small basophilic megaloblasts, and fairly numerous late megaloblasts. The twenty-four hour marrow showed small basophilic megaloblasts and intermediate cells, a few normoblasts and older megaloblasts. In the forty-eight hour aspiration the predominating erythroblasts were intermediate cells, pronormoblasts and normoblasts. Medium-sized polychromatophilic and older megaloblasts were still present in small numbers, and there was an occasional promegaloblast. It seems that there was still some megaloblastic regeneration made possible by the suboptimal medication. Figs. 19 and 20 were made from this forty-eight hour marrow. The six-day marrow was normoblastic except for an occasional late megaloblast.

The first patient received more adequate treatment, and marrow transformation was more rapid and complete than in the second patient. Even in the slowly transforming marrow of the second patient there was not the increase of typical older orthochromatic megaloblasts that would be expected if treatment caused an increased rate of maturation. Increasing the amount of liver injected hastens the marrow transformation but does not increase the number of older orthochromatic megaloblasts. This is illustrated in our Fig. 18, photographed at the same magnification as Figs. 19 and 20. This marrow was taken twenty hours after the injection of 15 units of liver extract. Megaloblasts are still present, but typical orthochromatic megaloblasts are scarce. Most of the erythroblasts are basophilic and polychromatic cells of intermediate structure; they are smaller than the cells of the marrow shown in Figs. 19 and 20, probably because of the rapid transformation of this marrow.

Another patient who was in relapse (hemoglobin 8.8 Gm. per 100 ml. of blood, erythrocytes 1,730,000 per cubic millimeter of blood) received 45 units of liver extract daily. Marrow was taken before treatment and forty-eight hours after the first injection. Before treatment his marrow contained many small promegaloblasts and basophilic megaloblasts with narrow cell body. The forty-eight hour marrow showed many normoblasts in all stages, but especially older, late polychromatophilic normoblasts. In the latter the cytoplasm had matured faster than the nucleus. In spite of this rapid transformation a few polychromatophilic and orthochromatic megaloblasts were seen, indicating continued maturation of the few polychromatic megaloblasts still present. However, there was no increase in the number of older megaloblasts, as claimed by Scott and by Rohr.[53, 54]

From these and other cases of our series we conclude with Jones,[32, 33] Fieschi[19, 20] and many others that maturation of megaloblasts present at the beginning of treatment continues during treatment. However, their number is not increased, for some degenerate, and promegaloblasts transform rapidly through numerous mitotic generations and do not produce any more typical megaloblasts. With suboptimal treatment megaloblastosis continues for a time, along with the formation of numerous intermediate cells and normoblasts, as illustrated in our Figs. 19 and 20.

In some cases suboptimal treatment may result in rapid transformation of the marrow, as illustrated in Fig. 21, which is from marrow taken forty-eight hours after the first daily injection of ½ U. S. P. unit of liver extract. The patient had an initial hemoglobin of 6.7 Gm. per 100 ml. of blood, erythrocytes

1,760,000, and leukocytes 2,700 per cubic millimeter of blood. The same amount of liver extract was injected daily for a time, and reticulocytes rose from 0.9 per cent to 20.5 per cent. The initial marrow was characterized by the presence of many small basophilic promegaloblasts, a few intermediate cells, and some normoblasts. The marrow picture was, therefore, not that of complete relapse. At twenty-one hours small basophilic promegaloblasts were still numerous. The intermediate cells had increased in number. At forty-eight hours (Fig. 21) there was a complete change of the marrow picture. Promegaloblasts and basophilic megaloblasts had disappeared, but polychromatic megaloblasts were fairly numerous, as were also myeloblasts and pronormoblasts. Most of the erythroblasts were of the types shown in Fig. 21. Some of these cells are quite normoblastic but others, especially the larger ones, are late intermediate forms. Gradations between the latter and normoblasts can be seen in the photograph.

The peak reticulocyte count of 20.5 per cent shows that the marrow of this patient was very responsive to small dosage of liver extract. However, the initial state of the marrow may have been the chief factor in the rapid transformation. Since some change toward normalization was evident before treatment, the patient might have been in the early stage of spontaneous remission in spite of the low hemoglobin and red cell count.

The patient of Figs. 19 and 20 received twice the dosage of liver given to the patient of Fig. 21, but his forty-eight hour marrow does not show the same degree of transformation.

E. The Intermediate Erythroblasts.—We have referred to our Figs. 18 to 21 as showing cells with nuclear structure that is intermediate between that of megaloblasts and normoblasts. Similar cells have been seen and described by a number of authors whose opinions regarding their significance have been summarized in our review of literature. These cells are the "érythroblastes intermédiaires" of Lambin and de Weerdt[38, 39] and the "Übergangszellen" of Rohr.[54] These authors and others we have mentioned regard these cells as true transitional stages between megaloblasts and normoblasts, the usual opinion being that the change is a gradual one through many generations of dividing cells. However, Davidson, Davis, and Innes (1942) believe in direct transformation of megaloblasts to normoblasts without intervening mitosis. Jones[34] classifies the cells with the megaloblasts as "atypical megaloblasts" and believes that they are not true transitional forms. Dacie and White also include them with the megaloblasts as "intermediate megaloblasts" and regard them as pathologic cells resulting from minor deficiency of hematopoietic principles.

We found the intermediate cells to be most numerous twenty to forty-eight hours after suboptimal treatment was begun. They are present after adequate therapy, as shown in Fig. 18 (twenty hours after injection of 15 units of liver extract), but the intermediate picture is not as conspicuous as when marrow conversion is slow (Figs. 19 and 20, forty-eight hours after injections of 1 U. S. P. unit of liver extract were begun). Fig. 18 shows greater progress toward normalization than is evident in Figs. 19 and 20, but megaloblasts are still

present. Polychromatophilic megaloblasts are seen in the upper right corner of Fig. 18 and in the upper central portion. The other cells are intermediate forms, some of which are quite normoblastic but not quite normal.

The intermediate forms tend to retain their nucleoli longer than do normoblasts of normal marrow. This is shown especially well in the slightly polychromatophilic cell in the upper left corner of Fig. 18. Except for the nucleoli the nuclear pattern of this cell is similar to that of basophilic or early polychromatophilic normoblasts.

Figs. 19 and 20 are two fields from the same film of concentrated marrow. Each field contains one large promegaloblast, and erythroblasts which show gradations of nuclear structure from promegaloblasts to cells that are quite normoblastic but not typical normoblasts. Fig. 19 is especially good for the intermediate forms. We recommend that they be examined with a reading glass. An atypical mitosis with scattered chromosomes, characteristic of many dividing megaloblasts, is also seen in this field. The color of the cytoplasm suggests that this cell is a polychromatophilic megaloblast.

Fig. 20 shows some small cells with pyknotic nuclei which probably are small normoblasts. Many of the small cells have nuclear buds, or dumbbell-shaped nuclei, like the one in the upper margin of the figure, indicating that many of the first definitive cells to appear after the beginning of treatment are not quite normal.

Complete division of nuclei without cell division of normoblasts and intermediate cells was seen several times in marrows during the early stages of conversion. Fig. 22 shows two cells with double nuclei which, according to the nuclear pattern, should be normoblasts. The cell below the promyelocyte is a polychromatophilic megaloblast, and the cell to the left of this one is a polychromatophilic intermediate erythroblast with nucleus that shows some normoblastic characters. The upper left-hand cell is close to the basophilic normoblast, although its nucleus is not quite typical of this stage.

Atypical mitoses, similar to those that have been described in erythroblasts of pernicious anemia marrow during extreme relapse (Fieschi[19, 20]), occur also in the normalizing marrows during the early stages of treatment. Examples are shown in Fig. 19, and the triple mitosis of Fig. 23. In the latter, two isolated chromosomes are to be seen, and one which forms a bud from the main chromatin mass. Isolated chromosomes and nuclear buds are also of rather frequent occurrence in megaloblasts whose nuclei are in the resting phase. The other cells of Fig. 23 are intermediate cells, the lower central one being more normoblastic than the others.

Figs. 22 and 23 are from the same film of concentrated marrow taken twenty-four hours after the patient had been given 5 mg. of folic acid by mouth and 5 units of liver extract parenterally. Before treatment his marrow contained many large promegaloblasts and basophilic megaloblasts with prominent nucleoli. The twenty-four hour marrow contained very few large promegaloblasts but had many smaller promegaloblasts and basophilic megaloblasts, and numerous intermediate cells. A few pronormoblasts and normoblasts were also seen.

As shown in our photographs, the intermediate cells have nuclei which exhibit gradations of chromatin pattern from that of the immature basophilic cells of the megaloblast series to the condensed characteristic arrangement of chromatin of normoblastic nuclei. The cells are usually reduced in size as the nucleus condenses, but in some instances a nucleus that is more or less normoblastic may be developed before there has been any great reduction in size. This is illustrated in our Fig. 21. This marrow was taken forty-eight hours after the first daily injection of ½ unit of liver extract. We have mentioned this patient earlier as one who showed excellent response to this small dosage, probably because the pretreatment marrow did not show the picture of complete relapse. In the pretreatment marrow the promegaloblasts and basophilic megaloblasts were small, and there were some intermediate cells, although the hemoglobin was 6.7 Gm. per 100 ml. of blood and the erythrocyte count was 1,760,000 per cubic millimeter of blood. Fig. 21 shows gradations of nuclear structure from "intermediate" to normoblastic without any change in the character of the cytoplasm.

After the beginning of treatment the first intermediate cells seem to be formed from the erythrogones, promegaloblasts and basophilic megaloblasts while they are dividing and being reduced in size. Usually there is considerable variation in the size of the cells as their nuclei develop the intermediate chromatin pattern (Figs. 19 and 20) and the cytoplasm becomes polychromatophilic. Since reduction in size of the earliest megaloblasts is one of the first changes noted after the beginning of treatment there are few of the largest basophilic erythroblasts present at this time.

Large groups of medium-sized transforming erythroblasts may show nuclear condensation and structure that is more or less normoblastic in a cytoplasm in which little or no hemoglobin has developed. This is illustrated in our Fig. 21, and in the photographs and colored figures published by Astaldi, Baldini, and Frugoni. The latter figures show that there has been great reduction in the size of the erythroblasts and condensation of their nuclei, while the cytoplasm has remained very basophilic. These figures are from twenty-hour and thirty-hour tissue cultures of pernicious anemia marrow after the patient received an injection of liver extract. The erythroblastic nuclei of the thirty-hour culture, while markedly condensed, are of intermediate structure rather than normoblastic, as stated by Jones and Smith (1950). The transformation toward normoblastic nuclei has progressed farther in our Fig. 21. The cytoplasm of all of these erythroblasts is in about the same stage of early polychromasia, although some nuclei are more normoblastic than others. These cells probably have been derived from medium-sized basophilic megaloblasts and promegaloblasts, which were very numerous at twenty-one hours.

The first intermediate cells usually appeared within twelve to twenty-four hours after the beginning of treatment and before the myeloblasts had become numerous. Myeloblasts and normoblasts were scarce in many of our pretreatment marrows, and these proved best for study of the origin of the first intermediate cells. In most cases we could not detect any considerable increase of myeloblasts earlier than forty-eight hours. Transitional stages between them

and normoblasts were noted, but we could not be certain that myeloblasts developed to intermediate cells, which might be numerous in the early hours while myeloblasts were scarce. However, in one case myeloblasts and transitional stages between them and normoblasts appeared twelve hours after injection of 15 units of liver extract, while intermediate cells were not numerous until twenty hours after injection. We could not see any relations between the early myeloblasts and the later-appearing intermediate cells.

These observations, our Figs. 18 to 21, and those published by Koller, Astaldi, Baldini, and Frugoni, and Rohr[54] are evidence in favor of the derivation of most of the intermediate erythroblasts of pernicious anemia under treatment from the immature basophilic primitive erythroblasts and youngest megaloblasts, rather than from the myeloblasts, which are not numerous in the early transformation stages. However, it is possible that some might be derived from myeloblasts as well as from free reticular cells (hemohistioblasts) and primitive erythroblasts (erythrogones), for Lambin and de Weerdt,[39] Jones,[34] Zuelzer and associates, and others have stated that the intermediate forms appear before the megaloblasts in early relapse. Koller expressed the belief that basophilic normoblasts also may produce intermediate cells and megaloblasts when relapse is developing, but that during the recovery phase the intermediate cells are formed from the youngest megaloblasts.

The intermediate cells also appear when there is partial deficiency of hematopoietic principle in marrows which become megaloblastic with greater deficiency, and in some refractory megaloblastic anemias (Dacie and White, Davidson, and Schartum-Hansen[57]). If there also should be shift to the left of erythropoiesis there would be primitive erythroblasts which could transform to the intermediate types.

The marrows of a few of our patients contained more than the usual number of myeloblasts, intermediate cells and normoblasts in addition to the megaloblasts, and so it can be assumed that these patients were in incomplete relapse. We have not had the opportunity to follow the early marrow changes of a progressive relapse.

By mitosis and differentiation intermediate cells produce generations of cells which gradually become normoblastic, and finally normoblasts. In the meantime, many of the intermediate cells differentiate to erythrocytes of large size. This results in the presence of polychromatic intermediate cells of various sizes and stages of differentiation. These cells are not necessarily derived from polychromatic megaloblasts, as claimed by Lambin and de Weerdt,[38, 39] and by Zuelzer and associates for some of them, or from orthochromatic megaloblasts as claimed by Pappenheim.[51] Completely orthochromatic erythroblasts cannot divide according to Wenderoth, who recently has made a careful study of this problem. We could not get any evidence that favored the direct transformation of megaloblasts to normoblasts claimed by Davidson, Davis, and Innes, who have stated that transformation is not the result of mitosis.

We agree with Lambin and de Weerdt,[38, 39] Jones,[34] Zuelzer and associates, Dacie and White, and others, that the "intermediate erythroblasts" are important constituents of the bone marrows of patients with megalocytic types

of anemia in whom there is a minor deficiency of the "liver factor." This includes patients in whom there never may be extreme deficiency, patients in the early stages of progressive relapse, and those in the early recovery phase from relapse following treatment. The intermediate cells may occur in the marrow at times when there are no megaloblasts and may thus be an important aid to diagnosis. Along with normoblasts the intermediate cells may be present in pretreatment marrow during relapse, but we cannot agree with Schartum-Hansen[57] that they are always present during relapse.

Until further study shows that similar cells occur in other types of anemia it might be best to refer to them as "atypical megaloblasts," as suggested by Jones,[34] or as "intermediate megaloblasts," as suggested by Dacie and White.

SUMMARY

Bone marrow aspirations were studied from two cases of sprue, three of hemolytic anemia, one with macrocytic anemia with normoblastic hyperplasia, several with normoblastic hyperplasia and uncertain diagnosis, and forty-five cases of pernicious anemia. The pernicious anemia patients were in complete or partial relapse, or in various stages of recovery following administration of optimal and suboptimal specific therapy. Suboptimal dosage gave the best material for study of the slow transformation of marrow from patients in complete relapse.

The primitive erythroblasts (erythrogones, proerythroblasts) of pernicious anemia are all of the same general type but vary in size, in basophilia of cytoplasm, and in size and character of nucleoli. Differences between them and the corresponding cells of normoblastic hyperplasia are no greater than between individual proerythroblasts of pernicious anemia. The most immature erythroblasts from both types of marrow can, therefore, be assumed to be identical. Both are derived from reticulum without going through a myeloblast stage, as in normal hemopoiesis.

Myeloblasts are scarce in some pretreatment marrows, and do not become numerous until some forty to forty-eight hours after the beginning of treatment.

Most of the large erythrogones and promegaloblasts are reduced in size in twelve hours after the beginning of treatment and begin transforming to intermediate erythroblasts in twenty to forty-eight hours, depending on treatment.

Intermediate erythroblasts usually appear before the myeloblasts become numerous.

Maturation of older megaloblasts continues after treatment is initiated, but the rate of maturation is not increased. Most of the immature basophilic promegaloblasts and erythrogones transform to intermediate erythroblasts, and these in turn to macrocytes or normoblasts. New promegaloblasts are no longer developed.

Older megaloblasts do not transform directly to intermediate erythroblasts or to normoblasts.

Intermediate erythroblasts are of clinical importance because their presence signifies a megaloblastic type of anemia, even though megaloblasts may not be

present at the time of examination, because of minor deficiency of hematopoietic factor.

Intermediate erythroblasts retain their nucleoli during maturation longer than cells of the normoblastic series.

Nuclei may change from intermediate pattern to normoblastic pattern without increase of hemoglobin beyond the early polychromatophilic stage.

Intermediate erythroblasts and the first normoblasts appearing after treatment may show abnormalities similar to those described for megaloblasts.

There is great variation in the rate of maturation of megaloblasts in pretreatment marrows. In a few cases there is little or no maturation arrest. Older megaloblasts are scarce in most pernicious anemia marrows when compared to the number of older normoblasts in normopoiesis.

Most of the first normoblasts to appear after treatment are derived from intermediate erythroblasts; others are derived from preexisting normoblasts, and from myeloblasts, especially after the latter have increased in number.

REFERENCES

1. Astaldi, G., Baldini, M., and Frugoni, C., Jr.: Transformazione Normoblastica del midollo osseo dell'anemia perniciosa ottenuta in vitro per azione diretta dell'estratto epatico, Haematologica. **31**: 265-300, 1948.
2. Astaldi, G., Tolentino, P., and Sacchetti, C.: La Talassemia (Morbo di Cooley e Forme Affini), Pavia, Tipographia del Libro, 1951.
3. Barta, I.: Die Bedeutung der Sternalpunktion bei Anämien und über die Beeinflussung des Knochenmarkes durch Leberbehandlung, Deutsches Arch. f. klin. Med. **171**: 565-577, 1931.
4. Berman, Lawrence, Jacobson, S. D., and Vonder Heide, E. C.: Observations on Response of Macrocytic Anemias to Folic Acid Therapy, Am. J. Clin. Path. **16**: 550-563, 1946.
5. Bilski-Pasquier, G.: Le problème du mégaloblaste, Ann. de méd. **49**: 417-440, 1948.
6. Chevallier, Paul: La maladie de Biermer, Sang. **6**: 359-401, 1932.
7. Chevallier, Paul: L'anémie, L'Expansion Scientifique Française 1948, 160 pp.
8. Dacie, J. V., and White, J. C.: Erythropoiesis with Particular Reference to Its Study by Biopsy of Human Bone Marrow: a Review, J. Clin. Path. **2**: 1-32, 1949.
9. Dameshek, William, and Valentine, Eleanor H.: The Sternal Marrow in Pernicious Anemia: Correlation of the Observations at Biopsy With the Blood Picture and the Effects of Specific Treatment in Megaloblastic (''Liver-deficient'') Hyperplasia, Arch. Path. **23**: 159-189, 1937.
10. Davidson, L. S. P.: Refractory Megaloblastic Anemia, Blood **3**: 107-120, 1948.
11. Davidson, L. S. P., Davis, L. J., and Innes, James: The Effect of Liver Therapy on Erythrocytopoiesis as Observed by Serial Sternal Punctures in Twelve Cases of Pernicious Anemia, Quart. J. Med. n.s. **11**: 19-27, 1942.
12. Doan, C. A.: Clinical Implications of Modern Physiologic Hematology, Beaumont Foundation Lectures, St. Paul, Minn., 1936, Bruce Publishing Co., 160 pp.
13. Doan, C. A., Cunningham, R. S., and Sabin, F. R.: Experimental Studies on the Origin and Maturation of Avian and Mammalian Red Blood-cells, Contributions to Embryology, Washington, Carnegie Institute, 1925, vol. 16, pp. 163-226.
14. Downey, Hal: The Myeloblast—Its Occurrence Under Normal and Pathological Conditions, and Its Relations to Lymphocytes and Other Blood Cells, Folia haemat., Arch. **34**: 65-89, 145-187, 1927.
15. Ellerman, V.: Untersuchungen über die Histologie der perniziösen Anämie, Virchows Arch. f. path. Anat. **228**: 247-275, 1920.
16. Fallon, Madeleine: Classification of the Anemias, Blood Pictures in the Anemias and Anemias in Infants and Children, in Downey, Hal: Handbook of Hematology, New York, 1938, Paul B. Hoeber, Inc., vol. 3, pp. 2147-2199.
17. Ferrata, Adolfo: Le Emopatie, Milano, Società Editrice Libraria, 1933-1935, 472 pp.
18. Ferrata, A., and Storti, E.: Le Malattie del Sangue, Milano, Società Editrice Libraria, 1948.
19. Fieschi, Aminta: Semeiologia del midollo osseo: Studio di morfologia clinica, Pavia, Tipografia già Cooperativa, 1938, 354 pp.
20. Fieschi, Aminta: Semiologie des Knochenmarks: Ein Studium klinischer Morphologie, Ergebn. d. inn. Med. u. Kinderh. **59**: 382-594, 1940.

21. Fieschi, Aminta, and Astaldi, Giovanni: La cultura in vitro del midollo osseo: Problemi di fisiopatologia ematologica studiati con la tecnica della cultura dei tessuti, Pavia, 1946, Tipografia del Libro, 309 pp.
22. Grawitz, Ernst: Klinische Pathologie des Blutes, Leipzig, 1911, Georg Thieme, 1011 pp.
23. Haenel, Ulrich: Über die Genese der megaloblastischen Blutbildung, Klin, Wchnschr. **26**: 8-12, 1948.
24. Helly, Konrad: Anämische Degeneration und Erythrogonien, Beitr. z. path. Anat. u. z. allg. Path. **49**: 15-33, 1910.
25. Henning, Norbert, and Keilhack, Heinz: Die Ergebnisse der Sternalpunktion, Ergebn. d. inn. Med. u. Kinderh. **56**: 372-460, 1939.
26. Introzzi, Paolo: Contributo allo studio della genesi dei megaloblasti nell'anemia perniciosa progressiva, e dei loro rapporti colle cellule fisse reticolari, Haematologica. **12**: 391-401, 1931.
27. Isaacs, Raphael: The Megaloblast as a Normal Stage in the Development of the Erythrocyte, Arch. Path. **14**: 286, 1932.
28. Isaacs, Raphael: The Erythrocytes. In Downey, Hal: Handbook of Hematology, New York, 1938, Paul B. Hoeber, Inc., vol. 1, pp. 13-14.
29. Israëls, M. C. G.: The Pathological Significance of the Megaloblast, J. Path. & Bact. **49**: 231-240, 1939.
30. Jaffé, R. H.: Erythropoiesis in Leukemia, Folia haemat. **49**: 51-63, 1933.
31. Jones, O. P.: Cytology of Pathologic Marrow Cells With Special Reference to Bone-marrow Biopsies. In Downey, Hal: Handbook of Hematology, New York, 1938, Paul B. Hoeber, Inc., vol. 3, pp. 2052-2071.
32. Jones, O. P.: Nature of the Reticulocytosis in Pernicious Anemia Following Liver Therapy, Proc. Soc. Exper. Biol. & Med. **38**: 222-226, 1938.
33. Jones, O. P.: Morphologic, Physiologic, Chemical and Biologic Distinction of Megaloblasts, Arch. Path. **35**: 752-775, 1943.
34. Jones, O. P.: Atypical Megaloblasts in Bone Marrows From Patients With Partial Deficiencies of Liver Principle (Abstr.), Anat. Rec. **94**: 473-474, 1946.
35. Jones, O. P., and Smith, A.: Transmission of Antianemic Principle Across Placenta and Its Influence on Embryonic Erythropoiesis; Comparison of Effect of Liver Extract and Pteroylglutamic Acid (PGA), Blood **5**: 499-521, 1950.
36. Koller, F.: Die Erythropoese bei der perniziösen Anämie mit besonderer Berücksichtigung der quantitativen Verhältnisse, Deutsches Arch. f. klin. Med. **184**: 568-626, 1939.
37. Lambin, Paul: Sur les modifications de la moelle osseuse dans l'anémie pernicieuse et leur signification pathogénique, Ann. Soc. Scient. de Bruxelles. **44** (pt. 1): 124-134, 1924-1925.
38. Lambin, P., and de Weerdt, W.: Le problème des mégaloblastes, Rev. belge sc. méd. **10**: 282-297, 1938.
39. Lambin, P., and de Weerdt, W.: Sur l'existence dans l'amémie pernicieuse d'un stade initial sans mégaloblastose médullaire, Sang. **13**: 928-932, 1939.
40. Leitner, S. J.: Bone Marrow Biopsy: Hematology in the Light of Sternal Puncture, New York, 1949, Grune & Stratton, 433 pp.
41. Limarzi, L. R.: Evaluation of Bone Marrow Concentration Techniques; a Modified Method for the Simultaneous Preparation and Staining of Blood and Bone Marrow Films, J. Lab. & Clin. Med. **32**: 732-740, 1947.
42. Limarzi, L. R.: The Effect of Arsenic (Fowler's Solution) on Erythropoiesis: A Contribution to the Megaloblast-normoblast Problem, Am. J. M. Sc. **206**: 339-347, 1948.
43. Limarzi, L. R., and Levinson, S. A.: Undescribed Type of Erythropoiesis Observed in Human Sternal Marrow, Arch. Path. **36**: 127-143, 1943.
44. Mallarmé, Jacques: The Study of the Myelogram (Bone Marrow Puncture) in Pernicious Anemia and the Problem of the Megaloblast, Blood **3**: 103-106, 1948.
45. Markoff, N. G.: Die Reticuloendothelien des Knochenmarks, beurteilt durch Sternalpunktion, Deutsches Arch. f. klin. Med. **180**: 530-538, 1937.
46. Naegeli, Otto: Blutkrankheiten und Blutdiagnostik; Lehrbuch der klinischen Hämatologie, ed. 5, Berlin, 1931, Julius Springer, 704 pp.
47. Nordenson, N. G.: Studies on Bone Marrow From Sternal Puncture, Esselte, Börtzells, 1935, 204 pp.
48. Nordenson, N. G.: Hematopoiesis From Sternal Puncture, Acta med. Scandinav. (Suppl.) **78**: 185-191, 1936.
49. Osgood, E. E., and Ashworth, Clarice M.: Atlas of Hematology, San Francisco, 1937, J. W. Stacey, Inc., 255 pp.
50. Pappenheim, Artur: Atlas der menschlichen Blutzellen. Supplement-Band. Jena, G. Fischer, 1912, pp. 204-209.
51. Pappenheim, Artur: Morphologische Hämatologie, Leipzig, Werner Klinkhardt, 1919, vol. 2, plate 8A.
52. Pappenheim, Artur: Haematologische Bestimmungstafeln, Leipzig, Werner Klinkhardt, 1920, 335 pp., plate IV.

53. Rohr, K.: Knochenmarksmorphologie des menschlichen Sternalpunktates, Neue dtsch. Klinik **14**: Ergänzungsband 4, 1937. Also in Klinische Fortbildung **4**: 498, 1937.
54. Rohr, Karl: Das menschliche Knochenmark: Seine Anatomie, Physiologie und Pathologie nach Ergebnissen der intravitalen Markpunktion, ed. 2, Stuttgart, 1949, Georg Thieme, 404 pp.
55. Sabin, F. R., and Miller, F. R.: Normal Bone Marrow. In Downey, Hal: Handbook of Hematology, New York, 1938, Paul B. Hoeber, Inc., vol. 3, pp. 1791-1838.
56. Schartum-Hansen, Hall: Zur Morphologie des Sternalpunktates bei perniziöser Anämie und makroblastischen Anämien, Folia haemat. **58**: 145-159, 1937.
57. Schartum-Hansen, Hall: The Sternal Marrow Function, With Special Reference to Erythropoiesis, in Pernicious Anemia, Acta med. Scandinav. (Suppl.) **218**: 7-276, 1948.
58. Schleicher, E. M.: Staining Aspirated Human Bone Marrow With Domestic Wright Stain, Stain Technol. **17**: 161-164, 1942.
59. Schleicher, E. M.: The Morphology of the Erythrogenic Reticulum of the Bone Marrow in Addison-Biermer's Disease in Relapse and Early Remission as Seen by the Imprint Method: With Reference to the Myelogenic and Megakaryocytogenic Reticulum, J. Lab. & Clin. Med. **30**: 928-943, 1945.
60. Schleicher, E. M., and Sharp, E. A.: Rapid Methods for Preparing and Staining Bone Marrow, J. Lab. & Clin. Med. **22**: 949-951, 1937.
61. Schulten, Hans: Zum Megaloblastenproblem, Folia haemat. **58**: 189-195, 1937.
62. Scott, R. B.: Sternal Puncture in the Diagnosis of Diseases of the Blood-forming Organs, Quart. J. Med. n.s. **8**: 127-172, 1939.
63. Segerdahl, Elsa: Über Sternalpunktion, Acta med. Scandinav. (Suppl.) **64**: 5-162, 1935.
64. Storti, Edoardo: Studio in vivo del midollo osseo nell'anemia perniciosa: Sulle primissime modificazioni morfologico-funzionali del tessuto mieloide conseguenti a epatoterapie, Haematologica **18**: 1-34, 1937.
65. Storti, E.: Diagnostica differenziale delle emopatie. In Ferrata, A.: Diagnostica medica differenziale, Milano, E. Sormani, 1939.
66. Veeneklaas, G. M. H.: Ueber megalozytäre Mangelanämien bei Kleinkindern, Folia haemat. **65**: 303-330, 1942.
67. Vogel, Peter, Erf, L. A., and Rosenthal, Nathan: Hematological Observations on Bone Marrow Obtained by Sternal Puncture, Am. J. Clin. Path. **7**: 436-447, 1937.
68. Wenderoth, H.: Cytoplasmaveränderungen in unreifen Blutzellen während der Teilung, Folia haemat. **69**: 116-135, 1950.
69. Witts, L. J.: The Pathology and Treatment of Anemia, Lancet **1**: 495-501, 549-557, 601-605, 653-657, 1932.
70. Young, R. H., and Osgood, E. E.: Sternal Marrow Aspirated During Life: Cytology in Health and Disease, Arch. Int. Med. **55**: 186-203, 1935.
71. Zuelzer, W. W., Newhall, Anne, and Hutaff, Lucile: Changes in the Bone Marrow in Megaloblastic Anemias of Infancy Before and After Folic Acid Therapy, J. Lab. & Clin Med. **32**: 1217-1230, 1947.

ADDITIONAL REFERENCES

(Not cited in text)

Ferrata, Adolfo, and de Negreiros-Rinaldi: Über die lymphoiden Vorstufen der hämoglobinhaltigen Normoblasten und Megaloblasten beim Embryo und beim Erwachsenen in normalem und pathologischem Zustand, Virchows Arch. f. path. Anat. **215**: 77-89, 1914.
Güse, T.: Über basophile Vorstufen der Megaloblastenreihe bei perniziöser Anämie, Deutsches Arch. f. klin. Med. **177**: 316-333, 1934-1935.
Hittmair, A.: Die Entstehung der Megaloblasten, Folia haemat. **69**: 234-238, 1950.
Kienle, F.: Ueber Knochenmarksfunktion im Lichte der Sternalpunktion, Folia haemat. **67**: 101, 1943.
Meyer, Erich, and Heincke, Albert: Über Blutbildung bei schweren Anämien und Leukämien, Deutsches Arch. f. klin. Med. **88**: 435-492, 1907.
Negreiros-Rinaldi: Morphologia normale e patologica dei globuli rossi, Napoli, 1916, Casa Editrice di "Folia Medica."
Piney, Alfred, and Hamilton-Paterson, J. L.: Sternal Puncture: A Method of Clinical and Cytological Investigation, ed. 3, New York, 1946, Grune & Stratton, 80 pp.
Schwarz, E.: The Nature of the Megaloblast, Hemat. Monographs, vol. 1, 1946.
Tempka and Braun: Das morphologische Verhalten des Sternumpunktates in verschiedenen Stadien der perniziösen Anämie und seine Wandlungen unter dem Einflusse der Therapie, Folia haemat. **48**: 355-401, 1932.
Weiner, W., and Kaznelson, P.: Über die zellige Zusammensetzung des Knochenmarkes nach Erfahrungen mittels der Sternalpunktion nach Seyfarth, Folia haemat. Arch. **32**: 233-261, 1926.
Ziegler, K.: Ueber die Morphologie der Blutbereitung bei perniziöser Anämie, Deutsches Arch. f. klin. Med. **99**: 431-467, 1910.

STUDIES ON PLATELETS. V. OBSERVATIONS ON THE REMOVAL BY THE PULMONARY CIRCULATION OF PLATELETS INJECTED INTO PATIENTS WITH IDIOPATHIC THROMBOCYTOPENIC PURPURA

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WITH THE TECHNICAL ASSISTANCE OF LUCY SALOMON

RESULTS of previous investigation[1] have shown that platelets disappear promptly when injected into the circulation of patients with idiopathic thrombocytopenic purpura (I.T.P.).[1] The implications of this finding for the interpretation of the pathogenesis of I.T.P. have been discussed at length.[1] Summarizing briefly, it may be said that prompt disappearance of injected platelets in I.T.P. could most likely be attributed to any or several of the following mechanisms: (a) removal and "sequestration" of platelets by various organs; (b) greatly increased utilization of platelets; or, (c) destruction of platelets in the peripheral circulation. The last-quoted hypothesis is supported by our findings,[1] the clinical observations of Evans and associates,[2] and the experimental results of Harrington and associates.[3] It should be mentioned, however, that I.T.P. is very likely a protein disease, where many different pathogenetic mechanisms might be at play in the individual patient.

All factors which might be responsible for the rapid disappearance of injected platelets in I.T.P. are being systematically investigated at present. Thus far we have focused our attention on the role of removal of circulating platelets by various organs in the pathogenesis of the thrombocytopenia of I.T.P. In view of the obvious although mysterious relationship of I.T.P. to the spleen, the ability of this organ to "sequester" platelets was investigated first, and our negative results are being reported.[4] The present communication is a critical study of the ability of the pulmonary circulation to remove platelets in patients with I.T.P. This investigation became necessary in view of the observations of Weisberger and associates[5] and of Bierman and co-workers[6, 7] on the role of the pulmonary circulation as a site of precipitous removal of leukocytes in man and experimental animals. Patients with I.T.P. were transfused with platelet-rich polycythemic blood, and the differences in platelet count between samples simultaneously collected from a vein of the arm and the common carotid artery recorded. With the limitations commented upon in the discussion, the results could be taken to give information on the removal of platelets during their pas-

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sage through the lung. In view of the potential danger of the procedure in patients with bleeding tendency, no attempt was made to use the more exact technique of intracardiac catheterization.

MATERIALS AND METHODS

The studies described in this paper were conducted in two young patients with I.T.P. of the "acute" variety.[1] Of these patients, Arthur P. made a spontaneous recovery, and the other, Timothy M., was splenectomized in view of the extreme severity of the bleeding manifestations a few days after our studies had been completed. Splenectomy was followed by recovery to date.

Under general ether anesthesia,* direct transfusion of platelet-rich polycythemic blood was started in the patient, utilizing a vein of the forearm. The technique of direct transfusion using completely Siliconized apparatus has been described in detail in a previous communication.[1] After at least 200 ml. of blood had been injected, and while the direct transfusion was being continued, needles were introduced in the cephalic or basilic vein of the arm other than the one where the blood was being administered and in one common carotid artery.† The direct transfusion was continued at a speed of approximately 20 ml. a minute; samples of blood were collected after the administration of approximately 15 ml. of polycythemic blood per kilogram of weight, and at five, ten, and fifteen minutes after the end of the direct transfusion. All needles were then removed, and a steady pressure applied for a few minutes over the site of the puncture of the common carotid artery. A small-sized hematoma formed in both cases, which receded in a few days without complications. Platelet counts in the peripheral blood were taken at regular intervals of time to establish the rate of disappearance from the peripheral circulation of the transfused platelets.

Silicone-coated 19-gauge needles were used for the collection of blood from the venous and arterial sides, a continuous very slow aspiration of blood into a glass syringe being maintained to prevent clotting of blood in the needles. When samples of blood were collected, Silicone-coated syringes were substituted and the necessary volume of blood was withdrawn with caution to prevent foaming. The blood was then transferred to a series of test tubes: (a) glass test tubes for the determination of the clotting time of whole blood, clot retraction, prothrombin activity of serum, and total and direct reacting serum bilirubin; (b) Silicone-coated test tubes from which samples were taken immediately for red cell, white cell, reticulocyte, and platelet[8] counts, hemoglobin determination, and for preparation of films for differential leukocyte counts; (c) Silicone-coated test tubes containing 1/10 volume of 0.1M of sodium oxalate for the determination of activity of prothrombin and P.P.C.F. (labile factor) of plasma, fibrinogen level, and fibrinolytic activity of plasma; (d) glass vial containing dry powder of balanced oxalate[9] for the determination of the hematocrit value. Most of the technique used in these studies has been described in previous communications.[10-12] The platelet count in the peripheral blood was obtained by the method of Dameshek.[8]

RESULTS

The results of this investigation are summarized in Table I and Fig. 1. It was confirmed that, when necessary precautions are taken,[1] a definite elevation of the platelet level may be achieved in patients with I.T.P. This, as shown previously,[1] is transient, the number of circulating platelets being back to the original value in the short period of thirty minutes to twelve hours. In our patients, both cases of the "acute" variety, the rate of disappearance of injected platelets was of forty-five minutes and seventy-five minutes, respectively.

*We are indebted to Dr. Robert N. Reynolds, Department of Anesthesiology, New England Center Hospital, for this procedure.

†By Dr. Vernon Marks, Department of Neurosurgery, New England Center Hospital.

Together with the rise in platelet count, the retraction of the clot and the utilization of prothrombin during the process of blood coagulation were normalized temporarily.

It was not possible to detect significant differences in the platelet count between samples taken from a brachial vein and the common carotid artery, at the time when polycythemic blood direct transfusion had just been completed and for some time afterward. In both cases studied it was found that a slightly higher platelet level could be found in the blood collected from the arterial side of the circulation. Although this finding is in agreement with the generally admitted observation that higher platelet count is found in the arterial than in the venous side, the differences recorded were well within the limits of experimental and technical errors, and were probably insignificant. Likewise, only minimal differences were noted in other blood constants studied.

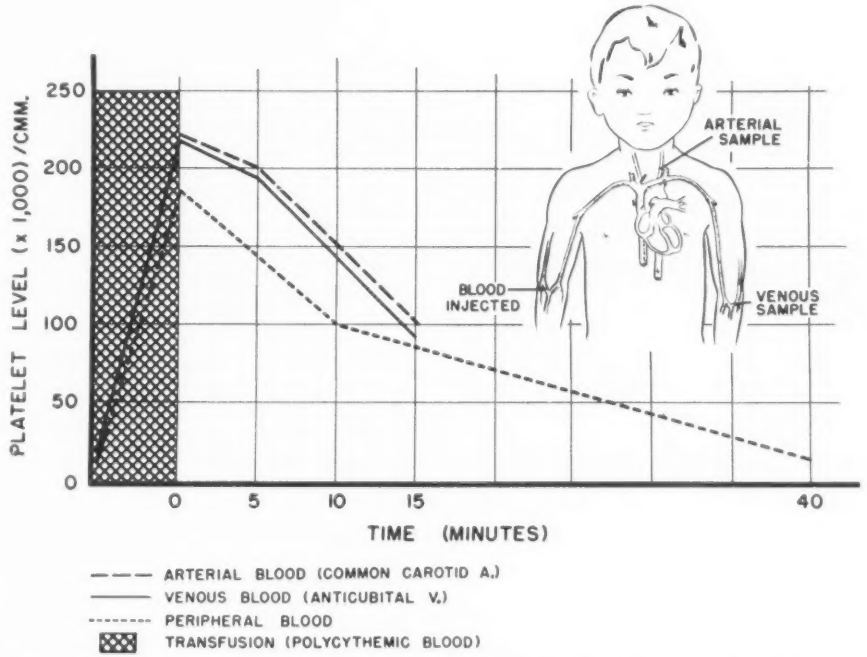

Fig. 1.—Diagrammatic representation of the study described in this article and comparison of the platelet count in venous, arterial, and peripheral blood of a patient with I.T.P. following the direct transfusion of platelet-rich polycythemic blood.

DISCUSSION AND CONCLUSIONS

The findings presented in this paper should be evaluated carefully in view of the limitations of the basic experiment involved. Our results, in this case, are obviously less significant and unequivocal than those previously reported with regard to the spleen.[4] In the latter case we were dealing with an organ with somewhat sluggish circulation; in the former with an organ through which large volumes of blood pass in the unit of time. This fact should be kept in mind when interpreting our findings.

TABLE I. THE BEHAVIOR OF VARIOUS CONSTANTS IN ARTERIAL AND VENOUS BLOOD OF PATIENTS WITH IDIOPATHIC THROMBOCYTOPENIC PURPURA RECEIVING PLATELET-RICH POLYCYTHEMIC BLOOD IN THE GENERAL CIRCULATION

	R.B.Cs. (MILLIONS /CU.MM.)	HEMOGLOBIN (GM./ 100 ML.)	HEMATOCRIT (PER CENT)	W.B.Cs. (/CU.MM.)	PLATELETS (X 1,000 /CU.MM.)	CLOT RETRACTION (PER CENT)	PLASMA PROTHROMBIN ACTIVITY (PER CENT)	SERUM PROTHROMBIN ACTIVITY (PER CENT)	P.P.C.F.* ACTIVITY (PER CENT)	FIBRINOGEN, MG./100 ML.
Case 1: Arthur, P. M., 4-year-old, given 15 ml. of polycythemic blood per kilogram of weight										
Donor's blood	4.95	12.4	44.6	14,300	2,524.5	†	90	0.3	90	171.4
Venous blood, before transfusion	3.47	11.2	31.9	8,100	34.7	7.9	90	68	100	399.6
Venous blood, end of transfusion	3.87	11.8	34.5	9,300	232.8	25.2	100	10	100	421.2
Venous blood, 5 minutes later	3.72	11.9	33.5	9,400	192.4	21.4	95	15	90	403.2
Venous blood, 10 minutes later	3.75	11.4	34	9,100	142.4	19.5	95	17.5	90	412.5
Venous blood, 15 minutes later			35		92.9					
Arterial blood, end of transfusion	3.74	12.0	35	7,500	238.8	21.4	95	9.5	100	414.7
Arterial blood, 5 minutes later	3.68	11.9	34.5	8,000	197.5	21.0	100	12.3	100	402.6
Arterial blood, 10 minutes later	3.74	11.9	34	8,400	151.4	20.5	100	14	100	400.7
Arterial blood, 15 minutes later					105.6					
Peripheral blood, end of transfusion	3.52	11.5		9,000	175.4					
Peripheral blood, 10 minutes later	3.47	11.7		8,200	102					
Peripheral blood, 45 minutes later	3.61	11.4		7,500	27.4					
Case 2: Timothy, M. M., 2-year-old, given 15 ml. of polycythemic blood per kilogram of weight										
Donor's blood	4.8	11.1	45.5	15,200	1,284	†	100	9.5	90	179.4
Venous blood, before transfusion	3.6	10.4	37	12,700	18	9.0	85	78	100	321.7
Venous blood, end of transfusion	3.8	12.1	37	21,000	169.2	21.5	95	31	90	301.4
Venous blood, 5 minutes later	3.7	11.8	38	18,500	121.7	19	95	37.5	100	307.2
Venous blood, 10 minutes later	3.8	11.5	38.5	19,000	110.2	17.5	95	42	100	299.0
Venous blood, 15 minutes later	3.6	11.2	37.5	20,500	81.3	16.5	95	64.5	100	303.7
Arterial blood, end of transfusion	4.0	12.4	40.5	16,600	182.4	24.0	100	25.5	85	297.2
Arterial blood, 5 minutes later	3.9	12.0	38.5	15,500	140.2	21.5	100	35.5	100	301.4
Arterial blood, 10 minutes later	4.0	11.8	40.5	16,500	120.4	18	90	42	100	298.9
Arterial blood, 16 minutes later	3.85	12.1	39.5	14,900	94	13.5	100	58.5	100	310.7
Peripheral blood, end of transfusion	3.9	11.8		17,000	137.5					
Peripheral blood, 15 minutes later	3.85	11.5		15,500	71.4					
Peripheral blood, 75 minutes later	3.79	11.7		16,200	22.5					

*Plasma prothrombin conversion factor (labile factor).

†Characteristic retraction of the clot, as seen in patients with polycythemic vera; the clot heavy with red cells and poor in texture sinks to the bottom of the tube.

Our results do not clearly suggest that the pulmonary circulation removes platelets actively in I.T.P., but their analysis also cautions against the conclusion that they exclude such a mechanism. Thus, in Case 1 we observed a drop of 140,000 platelets per cubic millimeter in a period of fifteen minutes, a loss of about 10,000 platelets per minute, or approximately 5,000 per cubic millimeter during each complete circulation of blood. In Case 2, the platelet loss was approximately 5,500 per cubic millimeter per minute. Should the lung remove platelets at such a slow rate, our present counting techniques would probably be inadequate to detect the differences in platelet count between the arterial and the venous side of the pulmonary circulation. We feel that, when critically interpreted, our findings may be taken to indicate that platelets disappear very quickly when injected into the peripheral circulation of patients with I.T.P., without showing conclusively whether their removal takes place in the peripheral circulation, the pulmonary circulation, or both. This conclusion might probably be interpreted as supporting our previously expressed view that removal of blood platelets in I.T.P. is not due to their "selective" precipitous sequestration in any particular circulatory district. A more important conclusion of our work is apparently that *the pulmonary circulation is unable to remove platelets as precipitously as white cells.*

With the accumulation of these negative findings, the mystery of the mechanism of injected platelets in I.T.P. remains unsolved. Several possibilities may be considered: (1) increased utilization of thrombocytes in the peripheral circulation; (2) destruction of thrombocytes in the peripheral vascular bed (presence of antiplatelet antibodies in the circulation of these patients?); (3) direct attack of platelets by circulating antibodies and, consequently, easier trapping and destruction of "sensitized" platelets by the spleen (?) and reticulo-endothelial tissue; (4) excessive splenic activity resulting in inhibition of production or release of thrombocytes from the bone marrow megakaryocytes. Any of these postulated mechanisms could be active in the individual case, the first and second likely being at play in the "acute" and the third in the "chronic" variety of the disease. Such possibilities are being systematically investigated at present.

SUMMARY

Samples of blood were collected simultaneously from common carotid artery and one brachial vein in two patients with idiopathic thrombocytopenic purpura of the "acute" variety, at the end and at various intervals of time after the direct transfusion of platelet-rich polycythemic blood into the peripheral circulation. The injected platelets disappeared quickly, but no significant differences in platelet level could be found at any time between the samples collected from the arterial and venous side of the pulmonary circulation. Although it cannot be excluded that active removal of platelets occurs in the lung, this obviously does not take place so precipitously as described for the white blood cells.

REFERENCES

1. (a) Stefanini, M., Chatterjea, J. B., and Dameshek, W.: Rate of Platelet Survival in Thrombocytopenia, J. Clin. Investigation **30**: 676, 1951 (abstract).
(b) Stefanini, M., Chatterjea, J. B., Dameshek, W., Zannos, L., and Perez-Santiago, E.: Studies on Platelets. II. The Effect of the Transfusion of Platelet-Rich Polycythemic Blood on the Platelet Count and the Hemostatic Function of Patients with Idiopathic Thrombocytopenic Purpura, Blood **7**: 53, 1952.

*Plasma prothrombin conversion factor (labile factor).
†Characteristic retraction of the clot, as seen in patients with polycythemic vera; the clot heavy with red cells and poor in texture sinks to the bottom of the tube.*

2. Evans, R. S., Takahashi, K., Duane, R. T., Payne, R., and Liu, C. K.: Primary Thrombocytopenic Purpura and Acquired Hemolytic Anemia: Evidence for a Common Etiology, A. M. A. Arch. Int. Med. **87**: 48, 1951.
3. Harrington, W. J., Minnich, V., Hollingsworth, J. W., and Moore, C. V.: Demonstration of a Thrombocytopenic Factor in the Blood of Patients with Idiopathic Thrombocytopenic Purpura, J. LAB. & CLIN. MED. **38**: 1, 1951.
4. Stefanini, M., Chatterjea, J. B., Dameshek, W., Welch, C. S., and Swenson, O.: Studies on Platelets. III. The Absence of "Selective Sequestration" and Destruction of Platelets by the Spleen in "Idiopathic Thrombocytopenic Purpura," Blood **7**: 289, 1952.
5. Weisberger, A. S., Heinle, R. W., and Hannah, R.: Transfusion of Leucocytes and Products of Disintegrated Leucocytes, Proc. Soc. Exper. Biol. & Med. **70**: 749, 1949.
6. Lanman, J. T., Bierman, H. R., and Byron, R. L., Jr.: Transfusion of Leukemic Leucocytes in Man; Hematologic and Physiologic Changes, Blood **5**: 1099, 1950.
7. Bierman, H. R., Byron, R. L., Jr., Kelly, K. H., Dod, K. S., and Black, P. M.: Studies on Cross Circulation in Man. I. Methods and Clinical Changes, Blood **6**: 487, 1951.
8. Dameshek, W.: A Method for the Simultaneous Enumeration of Blood Platelets and Reticulocytes, with Consideration of the Normal Blood Platelet Count in Men and Women, Arch. Int. Med. **50**: 579, 1932.
9. Wintrobe, M. M.: Clinical Hematology, ed. 3, Philadelphia, 1951, Lea and Febiger.
10. Stefanini, M.: New One-Stage Procedures for the Quantitative Determination of Prothrombin and Labile Factor, Am. J. Clin. Path. **20**: 233, 1950.
11. Stefanini, M.: The Diagnosis of Hemorrhagic Diseases, Bull. New England Med. Center **12**: 102, 1950.
12. Stefanini, M., and Crosby, W. H.: The One-Stage Prothrombin Consumption Test. Clinical Value in the Identification of Thromboplastin-Deficiency Diseases, Blood **5**: 964, 1950.

IRON ABSORPTION BY WOMEN: COMPARISON OF THREE FERROUS SALTS

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A NEED exists for specific information on the absorption, by human subjects, of iron from different iron salts which are used medicinally or in the enrichment of certain foods. Results of studies on rats and dogs are not completely applicable to man.

In man the absorption of iron from the gastrointestinal tract is normally controlled by his need for it. A person who is deficient in iron or who has an increased need, as in growth, pregnancy, and lactation, absorbs a greater proportion of the iron from the daily intake than does a normal person.

The study being reported was planned to compare the iron absorption and hemoglobin response of women given iron in the form of ferrous gluconate, ferrous lactate, or ferrous sulfate. Healthy nonanemic women were chosen for subjects in order to control in so far as possible the effect of iron need on iron absorption and thus reduce variability among the subjects. Eighty-three college students and staff members ranging in age from 18 to 42 years and living on self-selected diets served as subjects.

METHODS

For a period of four weeks, ferrous gluconate was given daily to twenty-seven of the women, ferrous lactate to twenty-nine, and ferrous sulfate to twenty-seven. The daily iron medication was: ferrous gluconate, 99 mg.; ferrous lactate 102 mg.; and ferrous sulfate, 101 mg. Capsules containing the iron salt were made especially for the study, and the maximum variation in the iron content of different capsules containing the same salt was 1 per cent, or 1 mg. The subjects took the capsules with the evening meal, and did not eat liver nor spinach during the month of medication.

Complete fecal collections were made during the third and fourth weeks of medication, that is, from the fourteenth through the twenty-eighth day.

The authors have had extensive experience with the analysis of biological materials for iron. Extreme precautions are routinely taken to avoid contamination and to insure recoveries between 98 and 102 per cent of quantitatively added iron (Leverton[1]). The fecal excretions of each subject for the two-week period were combined and made into a slurry with 10 per cent HCl, then an aliquot was ashed at 500° C., just below "red" heat. The white ash was dissolved in HCl, and the iron in it was determined by the method of Pohle and associates.[2] The optical density of the colored complex formed by 1,10-phenanthroline and the reduced iron was measured in a Beckman spectrophotometer for which the extinction coefficient of iron had been determined.

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Hemoglobin determinations were made in duplicate for two consecutive days before medication began and weekly thereafter until four weeks after medication had been discontinued. To avoid diurnal variation in hemoglobin values the venous blood samples for each subject were taken at the same time of day for each of the tests. Hemoglobin was converted to oxyhemoglobin in 0.1 per cent sodium carbonate, and the light transmittance of the solution was measured in the Sheard-Sanford photelometer.[3]

RESULTS

The mean daily intakes and absorptions of iron during the third and fourth weeks of iron medication are shown in Table I. The mean daily iron absorption by subjects given ferrous gluconate was 9 mg. (S.D. = 18.9), ferrous lactate 13 mg. (S.D. = 13.2), ferrous sulfate 11 mg. (S.D. = 10.0). The absorption was small because the need as evidenced by the normal hemoglobin values was small. Analysis of variance did not show a significant difference among these means.

For further analysis of the data the subjects were sorted into three groups on the basis of individual performance and the mean absorption of each group is also presented in Table I. Group 1 includes those who stored more than 5 mg. of iron daily; Group 2, those who excreted more than 5 mg. in excess of the amount they were ingesting; and Group 3, those who neither stored nor lost more than 5 mg. of iron daily and therefore were considered to be in equilibrium.

This rather generous interpretation of equilibrium allows for some of the vagaries of the digestive tract in the absorption and excretion of iron. Even among healthy individuals there are differences in gastric acidity, intestinal motility, and iron requirement which cause variation in iron absorption.

Sixty-three per cent of all the subjects were storing iron, 23 per cent were in equilibrium, and 14 per cent were losing iron from the body. The loss of iron by 14 per cent or twelve of the subjects when on daily intakes as high as 110 mg. of iron was unexpected, and is not easily explained. Diarrhea did not occur among these subjects.

Although only small differences in the absorption of iron from the three ferrous salts were observed, more subjects absorbed iron from ferrous lactate than from the gluconate or sulfate. Seventy-two per cent of the women given ferrous lactate stored iron and their mean daily absorption was 19 mg. (S.D. = 9.9). In contrast to this, only 52 per cent of the women given ferrous gluconate stored iron, however, their mean daily absorption was 24 mg. (S.D. = 13.6); and 63 per cent of the women given ferrous sulfate stored iron with a mean daily absorption of 16 mg. (S.D. = 8.3).

The means and standard deviations of the hemoglobin values for all subjects given each iron salt are shown in Table II. Hemoglobin values did not change significantly during the four weeks of the medication nor during the four weeks after medication had ceased. Nor was there any difference in the hemoglobin response of the subjects who were storing iron as compared with those in equilibrium or with those who were losing iron.

SUMMARY

Twenty-seven normal women were given ferrous gluconate, 29 ferrous lactate, and 27 ferrous sulfate at a level of approximately 100 mg. of iron daily

for four weeks. Iron absorption, or the difference between intake and fecal excretion, was measured during the third and fourth weeks of medication, and hemoglobin values were determined weekly during medication and for four weeks thereafter. The mean daily absorption of the subjects who were given ferrous gluconate was 9 mg., ferrous lactate 13 mg., and ferrous sulfate 11 mg. The difference between these means was not statistically significant. However, there was some indication that the women absorbed iron from ferrous lactate somewhat more consistently than from ferrous gluconate or ferrous sulfate. The hemoglobin of these normal women did not increase with iron medication.

TABLE I. MEAN DAILY INTAKE* AND ABSORPTION OF IRON DURING THIRD AND FOURTH WEEKS OF IRON MEDICATION

	FERROUS GLUCONATE		FERROUS LACTATE		FERROUS SULFATE	
	MEAN	S.D.†	MEAN	S.D.	MEAN	S.D.
All subjects	27		29		27	
Intake Fe (mg.)	109		112		111	
Absorption (mg.)	9	18.9	13	13.2	11	10.0
Group 1						
Subjects storing iron	14		21		17	
Per cent of subjects	52		72		63	
Absorption (mg.)	24	13.6	19	9.9	16	8.3
Group 2						
Subjects losing iron	8		3		1	
Per cent of subjects	29		10		4	
Absorption (mg.)	-11	6.4	-8	2.7	-6	0
Group 3						
Subjects in equilibrium	5		5		9	
Per cent of subjects	19		17		33	
Absorption (mg.)	-1	3.2	0	2.7	2	1.9

*The self-selected diets supplied approximately 10 mg. of iron daily.
†Standard deviation.

TABLE II. MEAN HEMOGLOBIN VALUES BEFORE, DURING, AND AFTER FOUR WEEKS OF IRON MEDICATION

	FERROUS GLUCONATE		FERROUS LACTATE		FERROUS SULFATE	
TIME OF TEST	MEAN	S.D.*	MEAN	S.D.	MEAN	S.D.
Number of subjects	27		29		27	
Initial† value, gm. per cent	13.4	0.65	13.2	0.73	13.1	0.69
Week of medication						
1	13.3	0.79	13.2	0.77	13.1	0.67
2	13.3	0.90	13.1	0.73	13.1	0.73
3	13.3	0.77	13.3	0.84	13.4	0.65
4	13.2	0.87	13.3	0.88	13.3	0.84
Week after medication						
1	13.4	0.69	13.2	0.75	13.3	0.83
2	13.5	0.84	13.5	0.91	13.3	0.66
3	13.4	0.93	13.4	0.91	13.3	0.88
4	13.4	0.82	13.3	0.76	13.4	0.69

*Standard deviation.
†Average of two values on consecutive days.

REFERENCES

1. Leverton, R. M.: Reliability of the Thiocyanate Method for the Determination of Iron in Acid Digests of Food and Feces, J. Home Econ. **30**: 252, 1938.
2. Pohle, W. D., Cook, J. H., and Mehlenbacher, V. C.: Determination of Iron in Foods and Food Products, Food Res. **12**: 229, 1947.
3. Todd, J. C., and Sanford, A. H.: Clinical Diagnosis by Laboratory Methods, Philadelphia, 1943, W. B. Saunders Company, p. 216.

THE RATE OF DISAPPEARANCE OF IODINATED HUMAN ALBUMIN FROM THE SERUM IN RELATION TO ALBUMIN CONCENTRATION, TOTAL CIRCULATING ALBUMIN CONTENT, AND THE PRESENCE OF ASCITES IN PATIENTS WITH CIRRHOSIS

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PATIENTS with portal cirrhosis have many metabolic abnormalities. Of particular importance is the disturbance in the synthesis and metabolism of the albumin fraction of serum. Previous studies have shown that the diminution in concentration of albumin in the serum of human patients may be correlated with the development of ascites—a complex disturbance which is influenced by many factors.[1-3]

The presence of ascites is a sign indicating a poor prognosis. Other aids to prognosis are the concentration of albumin in the serum and the total circulating albumin content, both of which depend upon the rate of formation of this protein.[1, 4] The ability of patients with cirrhosis to utilize albumin as a source of protein, as well as their ability to absorb other forms of protein, has been reported.[5-8] Previous studies, however, have not permitted observations on the rate of metabolism of albumin.

In the present study, using isotopic methods, iodinated human albumin was injected into patients with portal cirrhosis with and without ascites, and into control subjects.[9-11] The rates of disappearance from the vascular compartment in the three groups of patients were then compared.

MATERIAL

Twenty-three hospitalized individuals were studied. They were divided into three groups as follows:

Group 1 consisted of eleven subjects*—ten men and one woman—without clinical or laboratory evidence of liver disease. The ages ranged from 21 to 44 years. Nine had psychoneuroses, and two had epilepsy.

Group 2 consisted of seven patients—four men and three women—with enlargement of the liver and clinical evidence of cirrhosis; none had ascites. The ages varied from 23 to 56 years. In all these patients liver function tests† were abnormal and needle biopsies of the liver were diagnostic of portal cirrhosis. One subject was restudied after an interval of eight weeks, at which time he was improved. One patient died of a gastrointestinal hemorrhage twelve weeks after being studied, and permission for autopsy was not obtained. The remaining patients were living and showed clinical improvement; none have developed ascites.

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*The opportunity to study eight of the patients in the control group was made possible through the cooperation of the staff of the Veterans Administration Center, Mountain Home, Tenn., and Dr. E. C. Harper, Chief Medical Officer.

†Bromsulfalein retention, Van den Bergh, urine urobilinogen, cephalin-cholesterol flocculation, thymol turbidity, prothrombin time, and the excretion of hippuric acid after intravenous injection of sodium benzoate.

Group 3 consisted of five patients—four men and 1 woman—with portal cirrhosis and ascites. The ages ranged from 35 to 69 years. The four patients in whom liver biopsies were done had histologic findings which were diagnostic of cirrhosis. All had altered liver function tests. Four patients died two to eight weeks after being studied. One of these patients was restudied after a six-week interval of treatment two weeks before death. The remaining patient is clinically improved.

I^{131} TAGGED ALBUMIN*

The iodinated albumin was diluted with 250 ml. of sterile physiologic saline to give 1.5 to 3 μc of radioactivity per milliliter of solution to be injected. There was less than 1 Gm. of albumin in 20 ml. of the diluted solution.

The stability of the preparation was tested by dialyzing a highly radioactive solution of the iodinated albumin against distilled water at room temperature. At seventy-two hours, less than 1 per cent of the total activity was found outside the membrane.

METHODS

In preliminary studies on two patients with anarsarca, six blood samples were taken serially at five-minute intervals following the intravenous injection of 10 ml. of the iodinated solution and 10 ml. of T-1824 dye (Evans Blue).[12] It was found that both solutions reached a state of equilibrium throughout the vascular compartment at fifteen minutes, and showed no significant variation at the thirty-minute interval. The values obtained with the use of iodinated albumin were somewhat lower than those found with T-1824. In a similar study on eight patients without edema or ascites, it was found that equilibration of the iodinated albumin and T-1824 took place in ten minutes and was maintained for thirty minutes. These studies confirmed the results of others, who have found that plasma volume determinations using iodinated albumin tend to be smaller than those obtained with the dye T-1824.[13, 14]

The activity of all subjects was limited during the study. The control subjects were given a diet containing 90 to 100 Gm. of protein per day. The patients with cirrhosis were offered 120 to 130 Gm. of protein per day, and tube feeding was instituted when the intake was deficient. In the patients with ascites the salt intake was reduced to 0.8 to 2.0 Gm. per day. No attempt was made to restrict fluids. Diuretics and parenteral fluids were not administered before or during the study. Paracentesis was not done until after completion of the study, except for the withdrawal of 10 ml. of ascitic fluid from the patients in Group 3 for determination of radioactivity and albumin content. All patients were weighed daily during the study, and no significant variation was noted.

On the morning of the study each patient was weighed before breakfast. With the patient supine, 10 ml. of blood was withdrawn from an arm vein and used for determining the serum albumin concentration. The iodinated albumin solution was then injected into the same vein, from a calibrated sterile syringe. In Groups 1 and 2, 10 ml. was injected into each patient; in Group 3, 20 ml. was injected to facilitate counting.

Since it has been suggested that the plasma volume is frequently increased in patients with cirrhosis, a fifteen-minute period was allowed for the injected iodinated albumin to reach equilibrium throughout the vascular compartment in Groups 2 and 3.[4, 15] In the control patients (Group 1) only ten minutes was allowed to elapse. After ten minutes in the control patients and fifteen minutes in the patients with cirrhosis, the second blood sample (10 ml.) was removed from a vein in the arm opposite to the site of injection. Ten-milliliter samples were again withdrawn twenty-four, forty-eight, and seventy-two

*The tagged human serum albumin was obtained through the courtesy of Dr. D. L. Tabern of the Radioactive Pharmaceuticals Department of Abbot Laboratories, North Chicago, Ill.

hours after injection. All samples were allowed to clot in clean dry test tubes for at least one hour and then centrifuged at 3,000 r.p.m. for twenty minutes. One milliliter of the supernatant serum was pipetted into metal planchets and dried in air before being counted.

LABORATORY DETERMINATIONS

Physiologic Methods.—The *serum albumin concentration* was determined by the Kjeldahl method.

The *radioactivity* was measured by an autoscaler, using a shielded end-window Geiger tube in a fixed geometry. A standard consisting of 0.1 ml. of the diluted solution of iodinated albumin in 25 ml. of plasma was prepared for counting at the same time and in the same manner as the blood samples. All samples were counted together within ninety-six hours after the injection of the iodinated albumin. The activity of each sample was at least ten times background and was counted for a period sufficient to reduce the error to less than 2 per cent (a total of 4,096 counts).

In several patients in each group *serum radioiodide determinations* were made by the following method: two milliliters of serum, obtained at each of the various time intervals, was precipitated with 2 ml. of 20 per cent trichloracetic acid. After being allowed to stand for several hours in clean, dry test tubes, the precipitate was centrifuged for twenty minutes at 3,000 r.p.m. Two milliliters of the supernate was then transferred to metal planchets and its radioactivity determined. None of these samples showed significant activity.

In four of the patients in Group 3, the *radioactivity of the ascitic fluid* was determined. Ten milliliters of ascitic fluid was withdrawn twenty-four, forty-eight, and seventy-two hours after the injection of tagged human serum albumin. This fluid was prepared like the serum, and the radioactivity of 2 ml. samples was determined. Equilibration between ascitic fluid and serum was found to be present at twenty-four to forty-eight hours.

COMPUTATION OF DATA

The data were computed as follows:

1. Plasma volume $= \dfrac{\text{total activity injected (counts/milliliter)}}{\text{serum concentration 10 or 15 minutes after injection (counts/milliliter)}}$

2. Total circulating albumin (Gm./kilogram) = plasma volume (ml./kilogram) × serum albumin concentration (Gm. per cent)

3. Rate of disappearance of I^{131} albumin from vascular compartment at time interval X $= \dfrac{\text{concentration in serum at time interval X (counts/milliliter)} \times 100}{\text{total activity injected (counts/milliliter)}}$

STATISTICAL ANALYSIS

The mean values in the three groups were compared, and the mean and standard errors were calculated. The following formulas were used, in which sd = standard error, S^2 = pooled variants, n_1 = number of determinations in first group, n_2 = number of determinations in second group, and d = difference between the means. The value for t was considered significant when P = <.01. $sd = \sqrt{S^2 \dfrac{n_1 + n_2}{n_1 n_2}}$ $t = \dfrac{d}{sd}$

RESULTS (TABLES I AND II)

The *mean weight* and *plasma volume* (both absolute values and those corrected for body weight) did not differ significantly among the three groups.

When the *rate of disappearance of the tagged albumin from the vascular compartment* was compared in the three groups (Fig. 1), there was no sig-

nificant difference between means during the first forty-eight hours. The disappearance rates in the controls and in the cirrhotic patients without ascites were similar at forty-eight and seventy-two hours (79.80 ± 5.85 and 80.69 ± 2.89, respectively). In the patients with cirrhosis and ascites, however, the disappearance rate at forty-eight to seventy-two hours was significantly slower than those in groups 1 and 2 (t = 3.08 and 3.21, respectively).

The *serum albumin concentration* in both groups of cirrhotic patients was significantly reduced, as compared with the controls. The mean serum albumin concentration of the patients with ascites was also significantly lower than the mean value in the cirrhotic patients without ascites (2.14 ± 0.43 and 3.72 ± 0.82, respectively; P = < .01).

The mean *total circulating albumin* was significantly less in the patients with ascites than in groups 1 and 2 (t = 10.85 and 3.09, respectively). The difference between the cirrhotic patients without ascites and the control subjects was not significant.

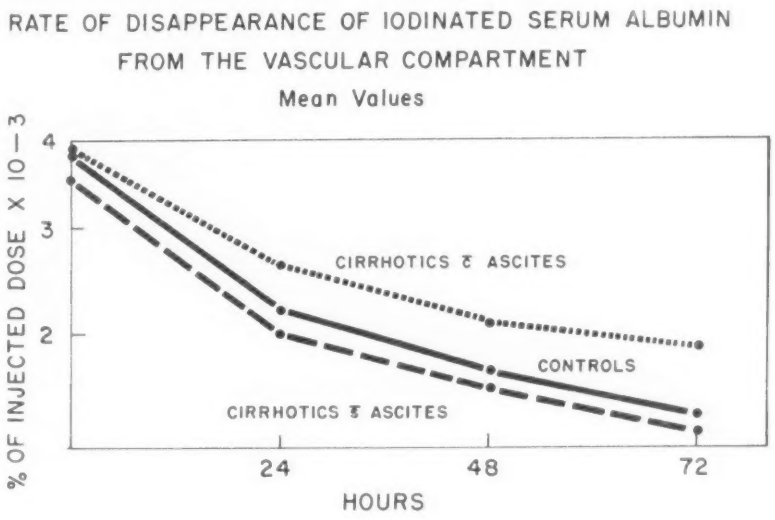

Fig. 1.

DISCUSSION

From the data, a correlation between rate of disappearance of iodinated albumin, serum albumin concentration, and total circulating albumin content appears to be present in the three groups of patients studied.

Rate of disappearance of iodinated albumin: When a negligible but detectable tracer amount of tagged albumin was injected intravenously, it reached equilibrium with the native albumin readily available to the circulation within a period of ten minutes if edema was not present. If anasarca was present, equilibrium was reached within fifteen minutes; a larger amount of tracer albumin was used in the presence of edema and ascites to facilitate counting. Once equilibrium was reached, it was maintained for at least thirty minutes indicating that the factor of dilution might be used as a measure of the total pool of al-

TABLE I. SUMMARY OF DATA

	GROUP 1				GROUP 2				GROUP 3			
	NO. OF DETERMINATIONS	MEAN	STANDARD DEVIATION	COEFFICIENT OF VARIATION	NO. OF DETERMINATIONS	MEAN	STANDARD DEVIATION	COEFFICIENT OF VARIATION	NO. OF DETERMINATIONS	MEAN	STANDARD DEVIATION	COEFFICIENT OF VARIATION
Weight (kg.)	11	69.7	8.1	11.6	8	67.6	18.9	27.9	6	78.6	19.5	24.8
Plasma volume (ml.)	11	2710	1740	64.2	8	3253	1307	40.2	6	2734	635	23.2
Plasma volume (ml./kg.)	11	38.8	6.5	16.8	8	48.1	14.8	30.8	6	35.6	8.4	23.6
Serum albumin (Gm. %)	11	5.62	0.69	12.3	8	3.72	0.82	22.0	6	2.14	0.43	20.1
Total circulating albumin (Gm./kg.)	11	2.15	0.30	14.0	8	1.82	0.84	46.2	6	0.74	0.17	23.0
Disappearance rate $\frac{24'}{10''\text{-}15''}$	11	60.37	4.84	8.0	7*	56.89	11.88	20.9	6	67.17	6.98	10.4
From vascular $\frac{48'}{24'}$	11	76.51	5.06	6.6	8	77.43	9.26	12.6	6	81.29	4.80	5.9
Compartment $\frac{72'}{48'}$	11	79.80	5.85	7.3	8	80.69	2.89	3.6	6	89.81	7.38	8.2

*In one individual the initial determination of the plasma volume was technically invalid. This determination was, therefore, repeated seventy-two hours later, and the plasma volume and total circulating albumin were calculated from the value.

bumin readily available for dilution with the blood, and hence as a measure of the plasma volume. That the ascitic fluid is not available for free exchange with the circulation is indicated by the fact that equilibrium with the ascitic fluid is not reached for twenty-four to forty-eight hours.

Previous studies with tagged albumin have shown the appearance of radioactivity in the urine of patients without albuminuria, suggesting that the iodinated albumin is being utilized by the body and the inorganic ion released.[16] This catabolism must proceed at a relatively slow rate, since no radioactive inorganic iodide was detected in the serum. The significantly slower rate of disappearance of radioactivity from the serum of patients with cirrhosis and ascites suggests that catabolism of this fraction proceeds more slowly in these patients with the poorest prognosis. In these patients the rate of formation of albumin, as judged by the total circulating albumin content, also appears to be impaired.

Serum albumin concentration: Our studies confirm those of others, who have demonstrated a decrease in serum albumin concentration in patients with cirrhosis.[17, 18] The fact that the concentrations were lowest in the patients with ascites indicates that the albumin fraction is of major importance in this complication. In this study no attempt has been made to evaluate the other factors known to be important in the pathogenesis of ascites—the pressure within the portal system, the relative electrolyte concentrations, and the effect of endocrine and lymphatic disturbances on the permeability of capillaries in the portal system.

Total circulating albumin content: It would appear that greater significance should be attached to the total circulating albumin content than to the concentration of this protein fraction within the serum. No attempt was made to evaluate the extent of edema formation, and hence the values for circulating albumin content could not be corrected for this factor.

Our data demonstrated no significant difference in the mean plasma volumes of the three groups. On inspection, the absolute values would suggest that the volume is increased in the group of cirrhotic patients without ascites, but sta-

TABLE II. SUMMARY OF STATISTICAL ANALYSES—COMPARISON OF THE MEANS IN THE THREE GROUPS

	WEIGHT (KG.)	PLASMA VOLUME (ML.)	PLASMA VOLUME (ML./KG.)	SERUM ALBUMIN (GM. %)	TOTAL CIRCULATING ALBUMIN (GM./KG.)	DISAPPEARANCE RATE		
						24′ ÷ 10″–15″	48′ ÷ 24′	72′ ÷ 48′
Group 1 compared with Group 2								
d	2.1	543	9.3	1.90	0.33	3.48	0.92	0.89
sd	6.3	436	5.0	0.35	0.28	3.97	3.29	2.26
t	0.33	1.25	1.86	5.43*	1.18	0.88	0.28	0.39
Group 1 compared with Group 3								
d	8.9	24	3.2	3.48	1.41	6.80	4.78	10.01
sd	6.6	295	3.7	0.31	0.13	2.87	2.52	3.25
t	1.35	0.08	0.86	11.23*	10.85*	2.37	1.90	3.08*
Group 2 compared with Group 3								
d	11.0	519	12.5	1.58	1.08	10.28	3.86	9.12
sd	10.3	580	6.8	0.37	0.35	5.54	4.17	2.84
t	1.07	0.89	1.84	4.27*	3.09*	1.86	0.93	3.21*

*Statistically significant; P = <.01.

tistical comparison does not bear out this impression. The differences are not significant when the plasma volume is calculated in terms of milliliters per kilogram of body weight. The lower total albumin content in the group with ascites, therefore, cannot be accounted for by alterations in the plasma volume. It appears that the total circulating albumin content is more directly related to the presence of ascites and to ultimate prognosis than is the serum albumin concentration.

Dynamics of Protein Metabolism.—The proteins of the body are in a dynamic state.[19, 20] Studies by the carbon-14 technique, using normal dogs, have demonstrated that the total amount of protein circulating in the blood is approximately equal to that in the extravascular fluid spaces.[21] Apparently there is a labile pool of protein on which the body can call for maintenance of homeostasis under various types of stress.

A reduction in the rate of formation of protein would alter the total circulating content. The amount of protein in the blood is apparently one factor affecting the function of organs and the distribution of fluids among blood, tissues, and organs. Of the various protein fractions, albumin is of greatest importance because of its power of osmosis and because of the apparent inability of damaged liver cells to form this fraction in adequate amounts. Judged by the lower rate at which the radioactively tagged albumin disappears from the blood of ascitic patients, the body attempts to conserve this protein when it is present in reduced amounts.

SUMMARY

Following the injection of tracer doses of iodinated human serum albumin, the rate of disappearance from the serum in relation to the albumin concentration and total circulating albumin content was determined in cirrhotic patients with and without ascites and in control subjects without liver disease.

In patients with cirrhosis and ascites the disappearance rate of the injected tagged protein was significantly slower, and the *total circulating* albumin was reduced. Such patients had a uniformly poor prognosis.

The data demonstrate that the presence of ascites in patients with cirrhosis is associated with a depletion of labile albumin stores.

REFERENCES

1. Post, J., and Patek, A. J., Jr.: Serum Proteins in Cirrhosis of the Liver. Relation to Prognosis and Formation of Ascites, Arch. Int. Med. **69**: 67, 1942.
2. Myers, W. K., and Keefer, C. S.: Relation of Plasma Proteins to Ascites and Edema in Cirrhosis of the Liver, Arch. Int. Med. **55**: 349, 1935.
3. Combined Staff Clinic: Mechanisms of Ascites Formation, Am. J. Med. **9**: 102, 1950.
4. Hiller, G. L., Huffman, E. R., and Levey, S.: Studies in Cirrhosis of the Liver. I. Relationship between Plasma Volume, Plasma Protein Concentrations, and Total Circulating Proteins, J. Clin. Investigation **28**: 322, 1949.
5. Thorn, G. W., Armstrong, S. H., Jr., and Davenport, V. D.: Chemical, Clinical and Immunological Studies on the Products of Human Plasma Fractionation. The Use of Salt-poor Concentrated Human Serum Albumin Solution in the Treatment of Hepatic Cirrhosis, J. Clin. Investigation **25**: 304, 1946.
6. Patek, A. J., Jr., Mankin, H., Colcher, H., Lowell, A., and Earle, D. P., Jr.: The Effects of Intravenous Injection of Concentrated Human Serum Albumin upon Blood Plasma, Ascites, and Renal Function in Three Patients with Cirrhosis of the Liver, J. Clin. Investigation **27**: 135, 1948.

7. Kunkel, H. G., Labby, D. H., Ahrens, E. H., Jr., Shank, R. E., and Hoagland, C. L.: The Use of Concentrated Human Serum Albumin in the Treatment of Cirrhosis of the Liver, J. Clin. Investigation **27**: 305, 1948.
8. Post, J., and Patek, A. J., Jr.: Serum Proteins in Cirrhosis of the Liver. II. Nitrogen Balance Studies on Five Patients, Arch. Int. Med. **69**: 83, 1942.
9. Fine, J., and Seligman, A. M.: Traumatic Shock. VII. A Study of the Problem of the "Lost Plasma" in Hemorrhagic, Tourniquet, and Burn Shock by the Use of Radioactive Iodo-plasma Protein, J. Clin. Investigation **23**: 720, 1944.
10. Crispell, K. R., Porter, B., and Nieset, R. T.: Studies of Plasma Volume Using Human Serum Albumin Tagged with Radioactive Iodine, J. Clin. Investigation **29**: 513, 1950.
11. Wasserman, K., and Mayerson, H. S.: Exchange of Albumin between Plasma and Lymph, Am. J. Physiol. **165**: 15, 1951.
12. Gibson, J. G., II, and Evelyn, K. A.: Clinical Studies of the Blood Volume. IV. Adaptation of the Method to the Photoelectric Microcolorimeter, J. Clin. Investigation **17**: 153, 1938.
13. Storaasli, J. P., Krieger, H., Friedell, H. L., and Holden, W. D.: The Use of Radioactive Iodinated Plasma Protein in the Study of Blood Volume, Surg., Gynec. & Obst. **91**: 458, 1950.
14. Aust, J. B., Chou, S. N., Marvin, J. F., Brackney, E. L., and Moore, G. E.: A Rapid Method for the Clinical Total Blood Volume Determination Using Radioactive Iodinated Human Serum Albumin (RIHSA), Proc. Soc. Exper. Biol. & Med. **77**: 514, 1951.
15. Perera, G. A.: The Plasma Volume in Laennecs Cirrhosis of the Liver, Ann. Int. Med. **24**: 643, 1946.
16. Tyor, M. P., Aikawa, J. K., and Cayer, D.: Measurements of the Disappearance of Radioactive Tagged Albumin from Serum and the Excretion of I^{131} in the Urine of Patients with Cirrhosis, Gastroenterol. May, 1952 (in press).
17. Cayer, D., and Nabors, G. C.: A Clinical Evaluation of the Total Blood Proteins and the Albumin and Globulin Fractions in Hospitalized Patients. An Analysis of Data in 300 Cases, South M. J. **40**: 744, 1947.
18. Tumen, H., and Bockus, H. L.: Clinical Significance of Serum Proteins in Hepatic Diseases Compared with Other Liver Function Tests, Am. J. M. Sc. **193**: 788, 1937.
19. Madden, S. C., and Whipple, G. H.: Plasma Proteins: Their Source, Production, and Utilization, Physiol. Rev. **20**: 194, 1940.
20. Schoenheimer, R., Ratner, L., and Rittenberg, D.: The Interaction of the Blood Proteins of the Rat with Dietary Nitrogen, J. Biol. Chem. **144**: 541, 1942.
21. Yuile, C. L., Lamson, B. G., Miller, C. J., and Whipple, G. H.: Conversion of Plasma Protein to Tissue Protein without Evidence of Protein Breakdown. Results of Giving Plasma Protein Labeled with Carbon-14 Parenterally to Dogs, J. Exper. Med. **93**: 539, 1951.

THE PATTERN OF ABNORMALITY OF LIVER FUNCTION IN METASTATIC CARCINOMA

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MEDICAL literature of the past three decades has numerous references to attempts to demonstrate the presence of metastatic carcinoma of the liver by the use of liver function tests. While a variety of tests have been used for this purpose,[3, 5, 6, 8, 10, 13, 15, 16] most observers have found the measure of the ability of the liver to excrete foreign dyes to be of the greatest value.[3, 5, 8, 13, 15, 16] The level of serum alkaline phosphatase has also been found frequently to be abnormal.[6, 10] Other tests of liver function, including serum bilirubin level, icterus index, hippuric acid excretion, and various carbohydrate tolerance tests, have been observed to be less consistently changed in the presence of hepatic metastases.[8a] The flocculation tests of liver function have been found to be infrequently abnormal under these circumstances.[8a]

In the present study, the results obtained with a small group of liver function tests, consisting of the bromsulfalein excretion, the thymol turbidity, and the cephalin-cholesterol flocculation tests, have been evaluated in patients with and without metastatic carcinoma of the liver.

MATERIAL AND PROCEDURES

Observations were made in fifty-three patients. In Table I are listed thirty-seven patients with carcinomatous metastases to the liver from various primary sites. In all of these cases the presence of metastases to the liver was confirmed by punch biopsy (B) exploratory laparotomy (L), or autopsy (A). In Table II are shown the seventeen patients with carcinoma in whom metastases to the liver were excluded by laparotomy (L) or autopsy (A).

In each patient a thymol turbidity test, cephalin-flocculation test, and bromsulfalein excretion test was performed in addition to the usual hospital physical and laboratory examinations.

All of these tests were done on the same morning. The thymol turbidity test was performed according to the technique of MacLagen[9] as modified by Shank and Hoagland[14] for the spectrophotometer. A reading of 4 units was considered as the upper limits of normal. The cephalin-cholesterol flocculation test was carried out according to Neefe and Reinhold's[12] modification of Hanger's[7] original technique. Only readings of 3 plus or 4 plus at the end of forty-eight hours were considered positive. Mateer and associates'[11] modification of the bromsulfalein test was performed using the 5 mg. per kilogram dose. The degree of dye retention was determined spectrophotometrically at the end of 45 minutes following the injection of the dye. Retention of 5 per cent or more at the end of 45 minutes was regarded as abnormal.

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TABLE I. DATA IN THIRTY-SEVEN PATIENTS WITH CARCINOMA AND METASTASES TO LIVER

PATIENT	1° SITE	METHOD ESTABLISHING METASTASES	BSP*	T. T.†	C. F.‡
N. C.	Stomach	L	18%	2.7	1+
E. M.	Stomach	A	6%	2.3	0
R. M.	Stomach	B	29%	10.8	4+
E. W.	Stomach	B	9%	1.0	3+
F. Y.	Stomach	B	22%	3.0	0
M. S.	Stomach	B	21%	3.1	1+
E. W.	Pancreas	B	21%	3.1	1+
P. H.	Pancreas	B, A	27%	3.1	1+
W. T.	Pancreas	L	35%	5.7	4+
L. B.	Pancreas	A	40%	7.8	3+
J. C.	Pancreas	B	22%	1.9	1+
F. C.	Colon	B	10%	2.0	0
R. B.	Colon	L	33%	3.2	4+
M. R.	Colon	L	18%	2.0	0
J. B.	Colon	A	30%	2.1	3+
A. McA.	Colon	B, A	23%	2.1	1+
R. K.	Colon	A	8%	0.8	0
C. R.	Esophagus	B, A	8%	1.4	2+
A. M.	Gall bladder	B, A	21%	2.4	1+
M. C.	Lung	B	22%	2.5	1+
D. B.	Lung	A	37%	8.0	1+
H. M.	Lung	A	12%	4.0	1+
J. DeN.	Lung	B, A	36%	1.1	4+
J. G.	Lung	B, A	29%	2.9	2+
W. S.	Lung	A	25%	1.4	3+
B. R.	Lung	L	26%	1.5	2+
A. D.	Breast	B, A	33%	3.0	1+
A. C.	Breast	B, A	30%	3.9	1+
L. G.	Breast	B, A	16%	2.1	0
M. M.	Breast	A	6%	4.0	1+
L. G.	Breast	L	55%	5.5	4+
T. C.	Seminoma	A	23%	2.8	3+
C. P.	Melanoma	A	41%	3.1	4+
A. F.	Melanoma	B	16%	2.1	3+
L. G.	Undetermined	B	8%	3.5	4+
M. L.	Undetermined	B	30%	3.2	3+
J. W.	Undetermined	B	24%	4.4	3+

*BSP, per cent bromsulfalein retention at forty-five minutes.
†T.T., thymol turbidity in units.
‡C.F., cephalin-cholesterol flocculation.

TABLE II. DATA ON SEVENTEEN PATIENTS WITH CARCINOMA BUT NO METASTASES TO THE LIVER

PATIENT	PRIMARY SITE	METHOD EXCLUDING METASTASES	BSP	T. T.	C. F.
H. A.	Stomach	A	9%	3.0	4+
A. V.	Stomach	A	2%	3.5	1+
T. G.	Stomach	L	4%	1.0	1+
R. T.	Stomach	L	3%	2.4	1+
A. M.	Stomach	L	13%	1.9	4+
L. H.	Stomach	L	10%	1.7	3+
F. B.	Esophagus	A	2%	3.2	2+
M. M.	Colon	L	3%	2.4	1+
E. T.	Breast	A	0%	0.8	1+
H. StL.	Lung	A	2%	1.3	3+
F. P.	Lung	A	2%	1.4	2+
C. McN.	Lung	A	10%	1.0	0
J. M.	Lung	A	12%	3.0	1+
N. L.	Undetermined	A	3%	1.9	1+
H. H.	Undetermined	L	3%	1.7	1+
T. H.	Cervix	L	0%	3.2	3+
W. P.	Synovioma	A	5%	0.9	0

TABLE III. LIVER FUNCTIONS IN PATIENTS WITH AND WITHOUT METASTASES TO THE LIVER

	NUMBER OF PATIENTS	ABNORMAL BSP RETENTION		ABNORMAL THYMOL TURBIDITY		ABNORMAL CEPHALIN FLOCCULATION	
		NUMBER	PER CENT	NUMBER	PER CENT	NUMBER	PER CENT
Metastases absent	17	6	35.3	0	0.0	5	29.3
Metastases present	37	37	100.0	8	21.6	15	40.5

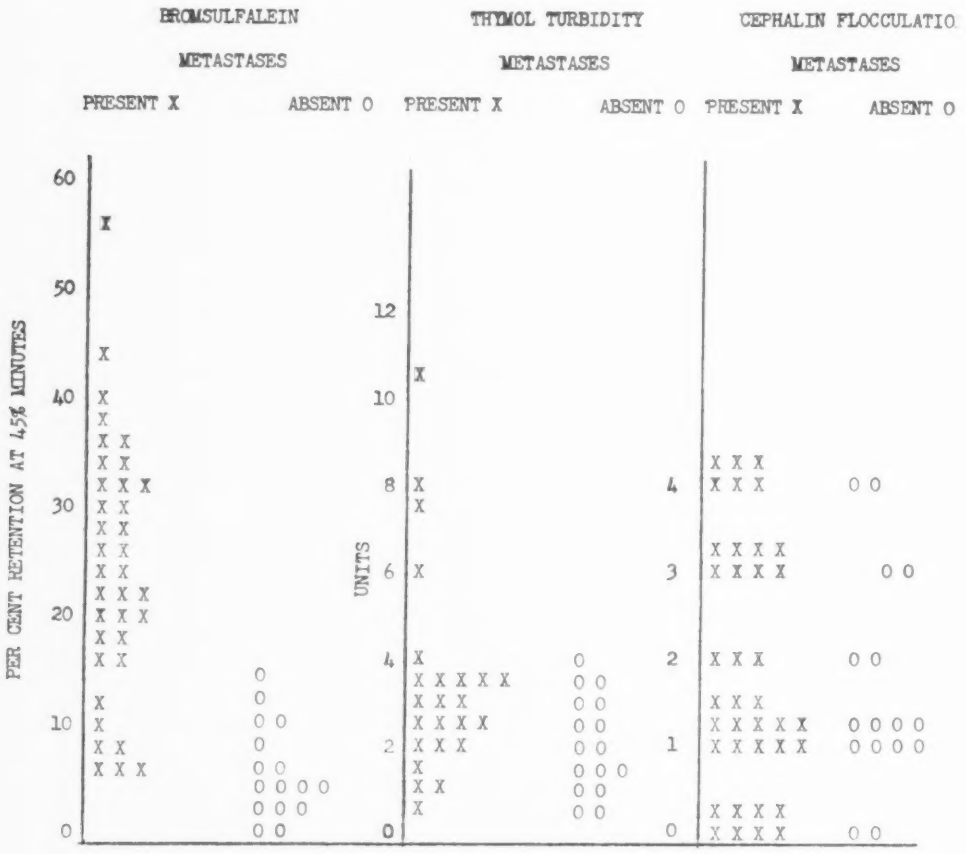

Fig. 1.—Scattergram showing the results with tests of liver functions in patients with carcinoma with and without metastases.

RESULTS

The data are shown in Tables I and II, and the results are summarized in Table III. All of the patients with metastatic carcinoma showed an abnormal degree of bromsulfalein retention. While bromsulfalein retention was also noted in some patients without metastases to the liver, it was present in a much smaller proportion of this group. Furthermore, the degree of abnormality of bromsulfalein excretion was frequently marked in patients with metastases, ranging from 6 to 55 per cent. On the other hand, it was relatively slight in the patients without metastases, ranging from 0 to 14 per cent (Fig. 1). The

results with the cephalin flocculation and thymol turbidity tests were, however, much less frequently abnormal in either group (Table III). Moreover, the difference between the two groups was much less striking when based on the flocculation tests than when the bromsulfalein results were considered. This pattern of disproportion between the distinct and frequently marked abnormality of bromsulfalein excretion and the normal or mildly abnormal flocculation tests was consistently noted in the patients with metastases, while in those without metastases the occasional slight abnormalities of all of the tests were relatively parallel in degree.

DISCUSSION

The impairment of the ability of the liver with metastatic carcinoma to excrete foreign dyes has been recognized since Greene and his co-workers evaluated the results with the phenoltetrachlorphthalein excretion tests. While most workers are in agreement that abnormal results with the bromsulfalein excretion test are frequent in the presence of metastatic carcinoma,[3, 5, 8, 13, 15, 16] the incidence of abnormality found in the present study is higher than that noted by others.[2] The high incidence may be due to the study of patients with more advanced metastatic involvement of the liver than that present in the patients studied by Ariel and Shahon.[2] Thus, while the frequency of abnormal bromsulfalein retention described here should not be interpreted to mean that all patients with metastatic carcinoma of the liver will have an abnormal bromsulfalein excretion test, it does show that the incidence of abnormality with this test is high. It seems of more importance to emphasize the coexistence of normal flocculation tests with abnormal bromsulfalein excretion, or the disproportion between the degree of abnormality of the two types of tests. Although this pattern of abnormality of liver function when seen should suggest the presence of metastatic carcinoma of the liver, it is seen in other clinical conditions. In congestive heart failure the degree of abnormality of bromsulfalein excretion is frequently much greater than the degree of abnormality of the flocculation tests, and distinctly abnormal bromsulfalein excretion frequently coexists with normal values for the thymol turbidity and cephalin flocculation tests.[4] Occasionally patients with Laennec's cirrhosis will also show a similar pattern.

It has been stated[8b] that any reduction of hepatic blood flow decreases the rate of bromsulfalein removal from the blood regardless of the state of functional activity of the hepatic cells. Extreme degrees of retention of the dye may be seen in severe congestive heart failure. It seems probable that the pattern of liver function described in metastatic carcinoma may be due to local circulatory impairment due to masses of metastases, which have produced relatively little change in the hepatic cells.

Abels and his associates[1] have reported that patients with gastrointestinal carcinoma show abnormal tests of hepatic function. This phenomenon has been considered to be independent of metastases[2] and associated with a metabolic effect of the primary neoplasm.[1] While in the present study also, even patients without metastases showed abnormality of liver function, the pattern of abnor-

mality was distinctly different in those with and without metastases. In the absence of metastases, abnormality when present involved two or all three tests proportionately. On the other hand, in the presence of hepatic metastasis, the incidence and degree of bromsulfalein retention were much higher than that of the thymol turbidity or cephalin flocculation tests. Consequently, in contrast to the observations of Ariel and Shahon[15] the incidence of abnormal bromsulfalein excretion was much higher in the group with metastases than in the one without evident metastatic involvement of the liver.

SUMMARY

The status of liver function was evaluated in fifty-three patients with carcinomas originating in various sites. Of seventeen patients without evident metastases only six showed abnormal bromsulfalein retention, while of thirty-seven patients with metastases all showed abnormal bromsulfalein retention. The thymol turbidity was abnormal in about one-fourth of each group, and the cephalin flocculation abnormal in 30 per cent of the patients without and 40 per cent of those with metastases. Disproportionate retention of bromsulfalein with a much lesser degree of abnormality of the flocculation tests is frequently but not invariably characteristic of carcinoma with metastases to the liver. The pattern is not usually seen in patients with carcinoma which has not metastasized to the liver. The significance of these results is discussed. Attention is drawn to the occurrence of this pattern in other clinical situations.

REFERENCES

1. Abels, J. C., Rekers, P. E., Binkley, G. E., Pack, G. T., and Rhoads, C. P.: Metabolic Studies in Patients with Cancer of Gastrointestinal Tract; Hepatic Dysfunction, Ann. Int. Med. **16**: 221-240, 1942.
2. Ariel, I. M., and Shahon, D. B.: Hepatic Dysfunction in Candidates for Abdominal Surgery, Especially in Patients with Cancer, Cancer **3**: 608-623, 1950.
3. Bargen, J. A., and Rankin, F. W.: Tests of Hepatic Function in Carcinoma: Their Value in Cases of Neoplasm of Colon with and without Metastasis to Liver, Ann. Surg. **91**: 225-232, Feb. 1930.
4. Evans, J., and Associates: To be published.
5. Greene, C. H., McVicar, C. S., Walters, W. and Rowntree, L. G.: Diseases of the Liver. IV. Functional Tests in Cases of Carcinoma of the Liver and Biliary Tract, Arch. Int. Med. **36**: 542-560, 1950.
6. Gutman, A. B., Olson, K. B., Gutman, E. B., and Flood, C. A.: Effect of Disease of Liver and Biliary Tract Upon Phosphatase Activity of Serum, J. Clin. Investigation **19**: 129-152, 1940.
7. Hanger, F. M.: Serological Differentiation of Obstructive from Hepatogenous Jaundice by Flocculation of Cephalin-Cholesterol Emulsions, J. Clin. Investigation **18**: 261-269, 1939.
8. Lichtman, S. S.: Diseases of Liver, Gall Bladder, and Bile Ducts, Philadelphia, 1949, Lea and Febiger. (a) page 742, (b) pages 229, 834.
9. MacLagan, N. F.: Thymol Turbidity Test; New Indicator of Liver Dysfunction, Nature, London **154**: 670-671, 1944.
10. Meranze, D. R., Meranze, T., and Rothman, M. M.: Serum Phosphatase as Aid in Diagnosis of Metastasis of Cancer to Liver, Pennsylvania M. J. **41**: 1160-1164, 1938.
11. Mateer, J. G., Baltz, J. L., Marion, D. F., and MacMillan, J. M.: Liver Function Tests; General Evaluation of Liver Function Tests, and Appraisal of Comparative Sensitivity and Reliability of Newer Tests, with Particular Emphasis on Cephalin-Cholesterol Flocculation Test, Intravenous Hippuric Acid Test, and Improved Bromsulphalein Test with New Normal Standard, J. A. M. A. **121**: 723-728, 1943.
12. Neefe, J. R., and Reinhold, J. G.: Photosensitivity as Cause of Falsely Positive Cephalin-Cholesterol Flocculation Tests, Science **100**: 83-85, 1944.

13. Paulson, M., and Wyler, C. I.: Multiple Tests of Hepatic Function in Gastroenteric Malignancy; Value of Bromsulphalein, Hippuric Acid and Van Den Bergh Reaction in Detecting Hepatic Metastasis, with Evaluation of Normality of Hippuric Acid Test, Ann. Int. Med. **16**: 872-878, 1942.
14. Shank, R. E., and Hoagland, C. L.: Modified Method for Quantitative Determination of Thymol Turbidity Reaction of Serum, J. Biol. Chem. **162**: 133-138, 1946.
15. Snell, A. M., and Magath, T. B.: Use and Interpretation of Tests for Liver Function; Clinical Review, J. A. M. A. **110**: 167-174, 1938.
16. Wever, G. K., Althausen, T. L., Biskind, G. R., and Kerr, W. J.: Liver Function in Hepatic and Extrahepatic Diseases; Results of Clinical Experience with 326 Cases, Am. J. Digest Dis. **2**: 93-99, 1935.
17. Zimmerman, H. J., and Thomas, L. T.: To be published.

CORTICOTROPIN THERAPY OF CHRONIC LIVER DISEASES

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THE use of corticotropin (ACTH) and cortisone in various liver diseases has been explored in a small group of patients. There is an ever-increasing literature on the use of ACTH and cortisone. Administration of these hormones to patients with liver diseases has been reported previously.[1-6] At least transient benefit was obtained in many patients. However, results have been temporary in most instances of chronic liver disease. Even temporary benefit was lacking in the experience of Butt, Comfort, Power, and Mason.[6] Though complete recovery has occurred in acute hepatitis, spontaneous healing is the usual outcome in most cases anyway. Sborov[5] presented data which tended to show that the liver function tests returned to normal more rapidly in treated than in control subjects.

Although many laboratory studies were carried out in the present study of a group of ten patients with chronic hepatic diseases, the findings of most interest to us were the blood bile acid levels in patients with chronic regurgitation jaundice and intractable pruritus. The pruritus decreased or disappeared when the bile acid level declined to or toward normal during ACTH therapy.

MATERIALS AND METHODS

Four patients had evidences of hepato-cellular dysfunction characteristic of Laennec's cirrhosis. Four patients had cholangiolitic (primary biliary) cirrhosis, and one patient had biliary cirrhosis as a result of a common bile duct stricture. One patient had a chronic hepatic dysfunction, but a definite diagnosis of cirrhosis was not established. One patient with pruritus secondary to Hodgkin's disease was also treated with ACTH. Each patient with the exception of one with hepatic coma was studied during a control period, during therapy, and after withdrawal of ACTH. Serum electrolytes, blood glucose, fractional serum bilirubin,[7] cephalin cholesterol flocculation,[8] thymol turbidity,[9] cholesterol and cholesterol esters,[10] total and fractional serum proteins,[11-13] urine urobilinogen,[14] urine coproporphyrin,[15] blood bile acids,[16] eosinophil counts, [17] and 17-ketosteroid excretion[18] were carried out (see Fig. 1 and Table 1).

CASE REPORTS

CASE 1.—N. (No. 802292), a 53-year-old white housewife, was first admitted March 30, 1949. Bloating and eructations began five years previously, and jaundice and pruritus began one year previously. Examination showed jaundice, excoriations, and an enlarged liver and spleen. An operation revealed a normal biliary tract and a cirrhotic liver. T-tube drainage of the common bile duct relieved the pruritus as long as drainage was maintained. On Dec.

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29, 1949, she was readmitted. The pruritus and jaundice remained unchanged. ACTH (25 mg. every six hours) was given intramuscularly from Jan. 4 to 15, 1950. During this time her weight did not vary significantly. At first her appetite increased markedly. By the end of the treatment period she was tired and weak. Pruritus disappeared on the seventh day. The blood bile acids fell from 2.1 to 0.8 mg. per 100 ml. Relief from itching persisted for one month. Another course of ACTH was given for seven days. The itching decreased this time but did not disappear. Bile acids fell from 2.3 to 1.2 mg. per 100 ml.

CASE 2.—Mc. (No. 814210), a 55-year-old white widow, was admitted in January, 1950, complaining of pruritus for four years and jaundice for two years. She had no biliary colic, but had some dull right upper quadrant aching pain for ten years. There had been no excessive use of alcohol. Peritoneoscopy with liver biopsy in 1948 established the diagnosis of cirrhosis. A laparotomy in 1949 revealed a normal biliary tract. T-tube drainage of the

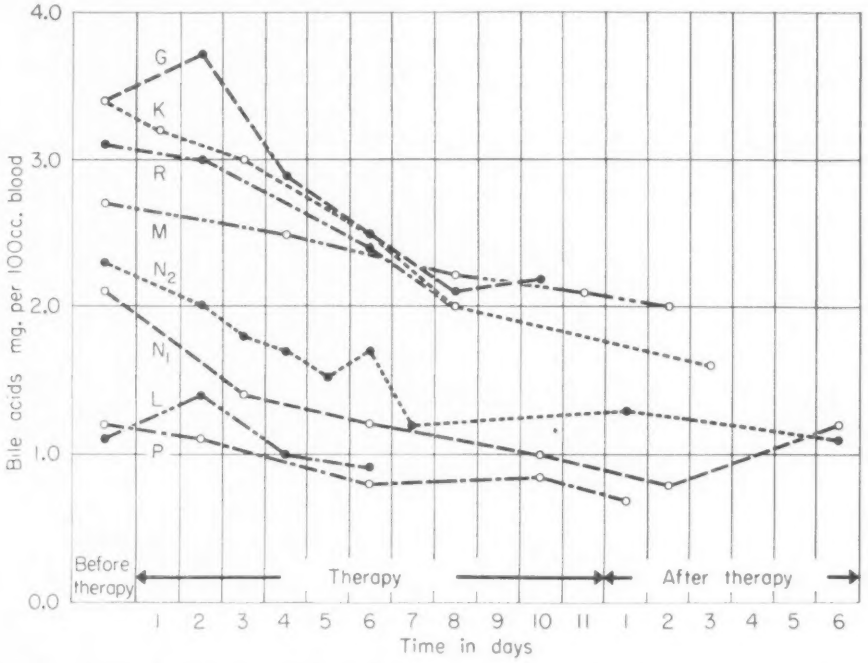

Fig. 1.—Blood bile acids. Normal blood specimens were examined each time a patient's blood was examined. The normal values ranged from 0.85 to 1.0 mg. per 100 ml. Patient L. had Hodgkin's disease and pruritus. Patient P. had Laennec's cirrhosis. The others had either primary or secondary biliary cirrhosis.

common bile duct at the time had resulted in complete relief from itching until removal of the tube. She was moderately jaundiced and had many excoriations of the skin. The liver extended 8 cm. and the spleen 2 cm. below the costal margins. ACTH (25 mg. twice a day for four days and then four times a day for eight days) was given intramuscularly from January 31 through February 11. Blood bile acids fell from 2.7 to 2.0 mg. per 100 ml. There was no improvement in pruritus during therapy.

CASE 3.—R. (No. 955372), a 39-year-old white housewife, was admitted to the hospital sixteen times from May, 1945, until May, 1950. She had pruritus of thirteen years' duration. She had had a laparotomy and T-tube drainage of the common bile duct in 1945, and had continuous external biliary drainage for two years with complete relief from pruritus. She also had osteomalacia of the pelvis and lumbar spine and Paget's disease of the pelvis and femur. Examination in May, 1950, revealed diffuse dark-brown pigmentation, multiple excoriations of the skin, enlarged hard liver, and a firm spleen. ACTH (20 mg. every six hours)

TABLE I. PERTINENT LIVER FUNCTION TESTS

NAME	BLOOD BILE ACIDS (MG./100 ML.)		SERUM BILIRUBIN (MG./100 ML.)		C.C.F.	T.T. (U./100 ML.)	Z.T. (U./100 ML.)	B.S.P.	U.U. (MG./DAY)	U.C.P. (γ/DAY)	SERUM PROTEINS (GM./100 ML.)			SERUM CHOLESTEROL (MG./100 ML.)	
	BEFORE	LOWEST	I'	T.							A.	G.	γG.	TOTAL	ESTERS
N.	2.1	0.8	8.0	13.1	2+	7	13		11.5	153	3.1	3.1	1.6	352	47%
M.	2.7	2.0	4.7	8.9	0	4	8		14.4	260	2.6	3.4	1.0	288	56%
R.	3.1	2.0	2.0	3.2	2+						2.2	5.7	2.7		
A. K.	3.4	1.6	1.5	3.2	2+	5	15			236	2.7	4.0		278	
G.	3.6	2.1	2.1	4.3		9	13	42%	4.6		3.0	4.3		248	62%
K.			2.0	3.5	3+	2	14	14%	8.6	332	2.0	4.3	1.6	117	64%
P.	1.2	0.85	0.1	0.4	3+	12	31	14%	4.6	146	2.4	5.0	2.3	163	73%
B.	0.8	0.8	14.0	26.0	4+	16	22		32.8	550	2.4	4.1	2.0	196	63%
Kr.			2.9	5.8	4+	4	15		13.0	545	2.5	3.9	1.9	47	50%
S.			0.1	0.2	4+	7	12			158	2.1	4.4	1.4	269	78%

C.C.F.—Cephalin cholesterol flocculation.
Z.T.—Zinc turbidity.
T.T.—Thymol turbidity.
U.U.—Urine urobilinogen.
U.C.P.—Urine coproporphyrin.
A.—Albumin.
G.—Total globulin.
γG.—Gamma globulin.
B.S.P.—Bromsulfalein.

was given intramuscularly for eight days. Her weight increased 4 kilograms. The pruritus was alleviated somewhat. During the last two days she complained of severe weakness. Blood bile acids fell from 3.1 mg. to 2.0 mg. per 100 milliliters.

CASE 4.—K. (No. 782790), a 70-year-old white housewife, had a cholecystectomy in 1946 and developed a common duct stricture. This stricture was repaired in 1947, but jaundice persisted. In July, 1950, she was hospitalized because of anorexia, nausea, and hematemesis. She had episodes of chills and fever, and moderately severe pruritus. She was icteric and thin. Her temperature was as high as 100° F. The liver and spleen were enlarged and firm. There was evidence of collateral venous circulation on the abdomen. Jaundice decreased somewhat during the control period. ACTH (20 mg. every six hours) was given intramuscularly for eight days. The pruritus disappeared on the fifth day of therapy, and did not return during the rest of the hospital stay. Blood bile acids decreased from 3.2 to 1.6 mg. per 100 ml. in eight days.

CASE 5.—G. (No. 816946), a 55-year-old white housewife, was admitted to the hospital in January, 1951. She was well until five years ago when she began to have pruritus. She became jaundiced and noted gnawing pain in the right upper quadrant of the abdomen, and had dark urine and light stools. An exploratory laparotomy was performed shortly after onset of jaundice, and the common bile duct and gall bladder were normal, but the liver was cirrhotic. Pruritus disappeared completely during a three-month period of T-tube drainage of the common bile duct, but it recurred soon after removal of the tube. Examination revealed mild jaundice, increased pigmentation of the skin, and many excoriations of the skin. The liver and spleen were easily palpated and were firm. She received ACTH (20 mg. every six hours) intramuscularly for twelve days. Pruritus decreased but did not disappear entirely. Blood bile acids decreased from 3.6 to 2.2 mg. per 100 ml. The fourth day after stopping ACTH she had a massive hemorrhage from esophageal varices (which were demonstrated later by x-ray) and required a number of transfusions. Her pruritus did not return for six weeks, but then rapidly increased to previous intensity.

CASE 6.—J. B. (No. 817919), a 60-year-old white farmer, was admitted to the hospital on April 21, 1950. He had noted an increasing girth and a 20-pound weight gain for six months, increasing pyrosis for three months, and jaundice and dark urine for two weeks. He had consumed one to eight bottles of beer daily for twenty years. Examination revealed jaundice, moderate obesity, marked fetor hepatis, ascites, and a firm, nontender liver extending 5 cm. blow the costal border. He was fed a low sodium diet. ACTH (20 mg. every six hours) was given intramuscularly for six days. Some confusion developed after three days. An original slight oliguria was followed by a diuresis on the third day. Following ACTH therapy ascites and edema increased. Gastrointestinal bleeding followed, and the patient expired on May 29, 1950. Autopsy revealed cirrhosis of the liver, pleural effusion and ascites, esophageal varices, massive hemorrhage from varices, infarction and fat necrosis of the tail of the pancreas.

CASE 7.—A. K. (No. 772877), a 53-year-old white laborer, was admitted to the hospital on Nov. 11, 1949, complaining of weakness and diarrhea for nine months. He had a subtotal gastrectomy for a benign gastric ulcer in 1946, and was found to have induration of the pancreas. Physical examination showed a chronically ill icteric male with a few spider nevi over the shoulders and chest. A firm nontender liver could be palpated 4 cm. below the costal border. The spleen was not palpable. A needle biopsy of the liver showed cirrhosis of the liver with very little fatty infiltration. ACTH (25 mg. every six hours) was given intramuscularly from Nov. 29 through Dec. 4, 1949. ACTH was stopped because of the development of confusion, delirium and semi-coma on the sixth day. The patient regained mental poise three days after stopping ACTH. Four months following discharge the patient had gained about 30 pounds of weight and was feeling quite well.

CASE 8.—L. K., a 43-year-old white housewife, was admitted to the hospital on April 14, 1950. She had gradually increasing ascites for two years. Paracentesis was followed by oliguria, jaundice, and rapid accumulation of ascites. She had taken alcohol in excess

for twenty years. She had fetor hepatis, ascites, hypertension, and an ulcer on the left leg. She lapsed into coma. ACTH (20 mg. every six hours) was started on May 5 and continued until the patient expired on May 10. The clinical status was unaffected. During the first three days her temperature did fall from 102° F. to normal, but fever recurred. Autopsy revealed cirrhosis, ascites, splenomegaly, left ventricular cardiac hypertrophy, pulmonary congestion, and hydrothorax.

CASE 9.—M. P. (No. 780400), a 36-year-old white housewife, was admitted to the hospital on July 6, 1947. She had noted weakness for four years, swelling of the abdomen for seven months, and diarrhea for three weeks. She denied any episode of jaundice, biliary colic, or of excessive use of alcohol. She had ascites, splenomegaly, and multiple cutaneous spider nevi. Biopsy proved the presence of cirrhosis of the liver. She was readmitted to the hospital on Feb. 1, 1950. Ascites had disappeared, but she had persistent anemia and neutropenia. ACTH (20 mg. every six hours) was given intramuscularly for twelve days starting March 8. The sodium content of the diet was restricted severely, but she gained 4 kilograms, and ascites reappeared. There was slight euphoria, but on the eighth day she developed anorexia and weakness which continued until three days after cessation of therapy. Blood platelets rose from 85,000 to 302,000 and the leukocytes from 1,300 to 3,500. Immediately after discharge from the hospital (within a week of stopping ACTH) she had her first hematemesis. Ascites and fetor hepatis which developed during ACTH therapy persisted. The platelets were 158,000 and leukocytes 3,750 one month later.

CASE 10.—M. S. (No. 817941), a 32-year-old white housewife, was admitted to the hospital April 22, 1950. She had albuminuria since pregnancy sixteen months previously. She had intermittent eczema, easy bruising, and food allergies for years. In 1946 a positive Wassermann reaction was noted. In 1949 a prolonged prothrombin time and a 4 plus cephalin flocculation test was found. Her blood pressure was 120/76. There was an eczematous rash over the cheeks, nose, and forehead, and there were many bruises and ecchymotic areas on the lower extremities. The urine had a maximal specific gravity of 1,024 and contained 2.7 Gm. of protein per liter, occasional red cells, white cells, and casts. Hemoglobin, leukocytes, platelets, and reticulocytes were normal. The prothrombin time was 31 seconds (control 14 seconds) by the one-stage Quick method, and the Lee-White clotting time was 1 hour and 41 minutes, but the bleeding time was normal. ACTH (20 mg. intramuscularly every six hours) was given for eight days. She was irritable throughout this treatment, but had no other mental changes. On a general diet she gained 3.5 kilograms. She felt weak and tired on the days following ACTH therapy, but soon returned to her previous status. Actually, no significant changes occurred in her status clinically or by laboratory tests.

CASE 11.—H. L. (No. 806174), a 42-year-old white man, had a six-year history of Hodgkin's disease. He had anemia and severe pruritus. He was emaciated. ACTH (20 mg. given intramuscularly every six hours) for ten days did not influence the pruritus or the course of his primary disease.

DISCUSSION

Ten patients with chronic hepatic disease and one patient with Hodgkin's disease and pruritus were treated with ACTH for periods varying from six to twelve days. Four of these patients had cholangiolitic primary biliary cirrhosis (N., Me., R., G.), and one patient (K.) had a stricture of the common bile duct and secondary biliary cirrhosis. These five patients had troublesome pruritus of many months or years duration. The other patients had either Laennec's cirrhosis or chronic hepatitis.

The blood bile acid concentrations were elevated in five patients to two to three times the upper limit of normal value (1.0 mg. per 100 ml.). In one patient (N.) the bile acid level fell to normal after twelve days of ACTH from 2.1 mg. per 100 ml. to 0.8 mg. per 100 ml. One month after cessation of therapy

the pruritus returned to its previous severity and the bile acid concentration rose to 2.3 mg. per 100 ml. A second course of ACTH therapy for seven days caused a drop in bile acids to 1.2 mg. per 100 ml. and nearly complete alleviation of the pruritus. Patient Mc. experienced only a slight diminution in pruritus. She had a fall in bile acid level from 2.7 mg. per 100 ml. to 2.0 mg. per 100 ml. in twelve days. Patient R. had only mild pruritus initially, and it decreased slightly during therapy. The bile acid level decreased from 3.1 mg. per 100 ml. to 2.0 mg. per 100 ml. in eight days. She had had severe pruritus for many years, but it had gradually diminished. Patient K. experienced complete cessation of pruritus, and the bile acid concentration decreased from 3.4 mg. per 100 ml. to 1.6 mg. per 100 ml. Patient G. noted nearly complete relief from pruritus, and the bile acids decreased from 3.6 to 2.1 mg. per 100 ml. The pruritus was relieved for six weeks, but then returned.

The patients with cirrhosis without pruritus were not benefited in any way by ACTH. One patient with thrombocytopenia, M. P., had normal platelets during therapy, but thrombocytopenia returned after cessation of ACTH therapy. There was no significant change in liver function tests. In seven of eight patients tested there was a decrease in gamma globulin concentration of the serum, and the average decrease was 0.4 Gm. per 100 ml. serum (from 1.55 Gm. per 100 ml. to 1.15 Gm. per 100 ml.). The average of total globulin decreased a corresponding amount from 4.1 Gm. to 3.74 Gm. per 100 ml.

There was no change in thymol turbidity measurements nor in the cephalin cholesterol flocculation tests. The zinc turbidity decreased during therapy in every instance where it was measured and paralleled the decrease in gamma globulin concentration. The serum bilirubin values decreased in some patients during and after therapy, but in others the serum bilirubin increased slightly. Fecal and urine urobilinogen and urine coproporphyrin determinations were carried out on six patients before, during, and after therapy. There was no consistent change.

There was a twofold to fivefold increase in 17-ketosteroid excretion in all but one patient, and in this patient there was a sixfold increase in the corticosteroid output (17-ketosteroid excretion was not measured in two patient). In all patients there was a striking decrease in circulating eosinophils during ACTH administration. This indicates that the adrenal cortices were stimulated during administration of ACTH.

Untoward effects were common. Two patients developed ascites and hematemesis, but recovered. One patient died of massive hemorrhage twenty-five days after cessation of therapy. It is difficult to be certain of a causal relation in this patient, but ascites increased, and progressive decline in health began during therapy. Several other patients complained of epigastric distress during therapy. Two patients noticed insomnia, and one patient became psychotic on the sixth day. Six of the patients were very tired starting about the seventh day of therapy.

One patient was comatose and moribund at the onset of therapy with ACTH. She became afebrile for a few days and lived for six days, but it is doubtful that ACTH changed the course in any way.

It is possible that the bile acids were used in the synthesis of adrenal corticoids. There was only a minor fall in bilirubin levels during therapy, so it is unlikely that the lowering of bile acid levels could be related to lessening of the regurgitation of bile.

SUMMARY AND CONCLUSIONS

Ten patients with chronic liver disease have been treated for short periods with ACTH. It is doubtful that any patient benefited significantly by this therapy.

Four patients developed serious complications. In one patient ascites increased during therapy, and the patient died of hemorrhage from esophageal varices twenty-five days later. Two other patients developed serious but nonfatal hemorrhage within the first week of withdrawal of therapy. One patient developed transient psychosis.

Four of five patients with elevated blood bile acid levels and pruritus noted partial or complete relief from pruritus. A significant lowering of blood bile acids occurred in these patients. The relief was temporary in each instance.

There was a significant decrease in serum globulins and especially gamma globulin in most of the patients.

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REFERENCES

1. Bongiovanni, A. M., and Eisenmenger, W. J.: Observations During the Administration of ACTH to Patients with Chronic Liver Disease, Proceedings of the Second Clinical ACTH Conference, Philadelphia, 1951, The Blakiston Company, Vol. I, pp. 390-397.
2. Colbert, J. W., Holland, J. F., Heissler, I., and Knowlton, M., The Use of ACTH in Acute Viral Hepatitis, Proceedings of the Second Clinical ACTH Conference, Philadelphia, 1951, The Blakiston Company, Vol. I, pp. 371-381.
3. Sborov, V. M.: Discussion of.[2] pp. 381-384.
4. Gyorgy, P., and Bluemle, L. W., Jr.: The Treatment of Chronic Inflammatory Liver Disease with ACTH and Cortisone, Proceedings of the Second Clinical ACTH Conference, Philadelphia, 1951, The Blakiston Company, Vol. I, pp. 386-389.
5. Bluemle, L. N., Sborov, V. M., Stokes, J., Jr., Gyorgy, P., and Neefe, J. R.: Use of Adrenocorticotrophic Hormone in Chronic Liver Disease (Three Cases), Proceedings of the First Clinical ACTH Conference, Philadelphia, 1950, The Blakiston Company, Vol. I, pp. 505-508.
6. Butt, H. R., Comfort, M. W., Power, M. H., and Mason, H. L.: Observations on the Effect of Cortisone Acetate on Two Patients with Hepatic Diseases, J. LAB. & CLIN. MED. **37**: 870-884, 1951.
7. Zieve, L., Hill, E., Hanson, M., Falcone, A. B., and Watson, C. J.: Normal and Abnormal Variations and Clinical Significance of the One-Minute and Total Serum Bilirubin Determinations, J. LAB. & CLIN. MED. **38**: 446-467, 1951.
8. Hanger, F. M.: Serological Differentiation of Obstructive from Hepatogenous Jaundice by Flocculation of Cephalin-Cholesterol Emulsions, J. Clin. Investigation **18**: 261-269, 1939.
9. Maclagen, N. D.: The Thymol Turbidity Test as an Indicator of Liver Dysfunction, Brit. J. Exper. Path. **25**: 234-241, 1944.
10. Schoenheimer, R., and Sperry, W. M.: A Micromethod for the Determination of Free and Combined Cholesterol, J. Biol. Chem. **106**: 745-760, 1934.
11. Majoor, C. H. L.: The Possibility of Detecting Individual Proteins in Blood Serum by Differentiation of Solubility Curves in Concentrated Sodium Sulfate Solution, J. Biol. Chem. **169**: 583-594, 1947.
12. Milne, J.: Serum Protein Fractionation, a Comparison of Sodium Sulfate Precipitation and Electrophoresis, J. Biol. Chem. **169**: 595-601, 1947.
13. Jaeger, B. V., and Nickerson, M.: A Simple Quantitative Chemical Method for Estimating Gamma-Globulin in Human Serum, J. Biol. Chem. **173**: 683-690, 1948.

14. Watson, C. J., Schwartz, S., Sborov, V., and Bertie, E.: Studies of Urobilinogen V. A Simple Method for the Quantitative Recording of the Ehrlich Reaction as Carried Out with Urine and Feces, Am. J. Clin. Path. **14**: 605-615, 1944.
15. Schwartz, S., Zieve, L., and Watson, C. J.: An Improved Method for the Determination of Urinary Coproporphyrin and an Evaluation of Factors Influencing the Analysis. J. LAB. & CLIN. MED. **37**: 843-859, 1951.
16. Josephson, B.: The Determination of Cholic Acids in Blood, Biochem. J. **29**: 1519-1524, 1935.
17. Randolph, T. G., and Gibson, E. B.: Enumeration and Differentiation of Leukocytes in the Counting Chamber with Propylene Glycol Aqueous Stains, Proc. Soc. Exper. Biol. & Med. **52**: 20-22, 1943.
18. Callow, N. H., Callow, R. K., and Emmens, C. W.: Colorimetric Determination of Substances Containing the Grouping CH_2CO in Urine Extracts, Biochem. J., **32**: 1312-1331, 1938.

THE EFFECTS OF CORTISONE ON THE CUTANEOUS HISTAMINE RESPONSE OF RABBITS USING EVANS BLUE DYE AS AN INDICATOR

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ADRENALECTOMY produces a marked decrease in resistance to systemic histamine intoxication in experimental animals, and administration of adrenal steroids partially restores resistance.[1] Studies of the effects of adrenal cortical activity on cutaneous histamine response have produced negative results, however. Adrenalectomy does not alter the cutaneous trypan blue staining response in rats,[2] and cortisone and ACTH administration to patients have failed to modify the cutaneous histamine wheal.[3]

Adrenal cortical inhibition of a number of tissue responses has been observed. Cortisone and ACTH suppress the inflammatory response in acute infections, chemical irritation, wound healing, the Shwartzman phenomenon, and serum sickness.[4, 5] In the latter, microscopic observation of living tissue reveals that cortisone maintains normal vascular tone and reduces endothelial damage.[6] Using intravenous administration of a colloidal dye to indicate changes in capillary permeability, adrenal steroids and/or ACTH have been observed to inhibit permeability increases due to exudate fluid and leukotaxine,[7] peptone,[8] hyaluronidase,[9] and carbon arc irradiation.[10]

In view of the possible role of histamine in mediating the above tissue responses, and the beneficial clinical response of hypersensitive states to cortisone and ACTH, the effects of cortisone on histamine response have been investigated further using Evans Blue dye as an indicator.

METHOD

The response to intradermal injections of histamine was tested forty-four times in a total of thirty animals. Treated animals received 10 mg. cortisone* daily for varying periods prior to testing, and in each experiment a number of normal animals served as untreated controls. The dosage schedules are given in Tables I to IV. The usual schedule was 10 mg. of cortisone daily for four days prior to testing. In Experiment 1*B* the treated and control groups of Experiment 1*A* were reversed two weeks after the initial testing. For this second part of Experiment 1 animals treated in the first part became the untreated controls, and the untreated animals in the first part were treated for four days prior to testing the second time.

Fresh solutions of histamine phosphate in normal saline were prepared in concentrations of 1.0, 0.1, and 0.01 mg. of free base per milliliter. In Experiments 1 and 2 concentrations of 0.001 and 0.0001 mg. per milliliter were also used, but it was learned that they were not useful for the purposes of the experiment. For a control solution either normal saline was used, or normal saline to which monobasic sodium phosphate was added to give a phosphate

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*Merck—Cortone Acetate; saline suspension.

TABLE I. STAINING RESPONSES OF UNTREATED AND CORTISONE-TREATED RABBITS TO INTRADERMAL HISTAMINE* 10 MG. CORTISONE DAILY FOR FOUR DAYS

	UNTREATED				CORTISONE TREATED			
EXPERIMENT & ANIMAL	CONTROL SALINE	HISTAMINE, MG./ML. 1.0	0.1	.01	CONTROL SALINE	HISTAMINE, MG./ML. 1.0	0.1	.01
Experiment 1*A*								
6	0	++++	+++	++				
12	0	++++	+++	+				
132	0	++++	+++	+				
1-b					0	0	0	0
2-b					0	0	0	0
3-b					0	0	0	0
Experiment 1*B*								
1-b	0	+++	++	+				
2-b	0	++++	+++	++				
3-b	0	++++	+++	++				
6					0	0	0	0
12					0	0	0	0
132					0	0	0	0
Experiment 2								
4	0	+++	+++	+				
7	0	+++	++	+				
140	0	++++	+++	++				
127					0	+	+	0
128					0	0	0	0
136					0	0	0	0
Experiment 3								
19	0	++++	+++	++				
20	0	+++	++	++				
21	0	++++	+++	++				
13					0	0	0	0
14					0	0	+	+
15					0	++	0	+

concentration equivalent to that of the strongest concentration of histamine. In one experiment pH measurement gave values ranging from 5.5 for the most dilute to 4.8 for the most concentrated histamine solutions, with pH 4.6 for the phosphate control solution.

Testing of staining responses was done on the day following the last injection of cortisone except in Experiment 5 (Table III). Using a No. 26 needle, intradermal injections of 0.2 ml. of each of the histamine solutions and a control solution were made in the animal's abdomen, which had been shaved several days before. Immediately after completion of the intradermal injections, Evans Blue dye was rapidly administered in the ear vein. In Experiment 2 the dye was given five minutes before the intradermal injections. The staining appeared to be more vigorous when the intradermal injections were given first.

In the first three experiments fresh solutions of Evans Blue dye in normal saline were prepared from the crystalline dye. Ten milliliters containing 8 mg. of dye were administered. In subsequent experiments 1.6 ml. of a commercial dye preparation* containing 5 mg. Evans Blue per milliliter of water was injected. After one batch of this preparation had been kept at room temperature approximately three months, it gave poor staining to complete lack of staining in untreated control animals. A fresh batch of dye has given good staining in control animals for six weeks. The new batch was found to diffuse more rapidly in gelatin than the old. It is probable that in this one instance, at least, the colloidal properties of the dye changed over a three-month period. Fresh dye was used in the experiments reported here, and good staining was obtained in all control animals.

*Warner—Experimental preparation, T-1824, Evans Blue dye.

RESULTS

At injection sites of the highest concentration of histamine blue halos developed in untreated animals given Evans Blue. The halos appeared within a few minutes at the margins of the injection blebs, and grew peripherally, reaching full development within thirty minutes. Within the halos the blebs showed early pallor, which was later replaced by staining. At sites of injection of the lowest concentration of histamine no halos appeared, but central staining of the blebs appeared early, within a few minutes. The intermediate concentration of histamine produced an intermediate type of response which often showed both a halo and early central staining. The halo was smaller than that produced by the highest concentration of histamine, and the central staining was often not as early or as intense as that produced by the lowest concentration. No true whealing was observed. Control solutions produced staining at stickpoints only. The responses were graded according to size and intensity of staining, the grades ranging between one plus for a light central stain to four plus for a dark halo measuring approximately 2 centimeters.

As shown in Table I (Experiments 1 to 3) all untreated animals responded with good staining. On the other hand, 75 per cent of the cortisone-treated animals in these experiments stained not at all, and the remainder stained poorly. That the difference in response was independent of individual differences in animal responsiveness was shown conclusively in Experiment 1. Tested

TABLE II. EXPERIMENT 4. STAINING RESPONSES OF UNTREATED AND CORTISONE-TREATED RABBITS TO INTRADERMAL HISTAMINE* 10 MG. CORTISONE DAILY FOR ONE- AND TWO-DAY PERIODS

TREATMENT	ANIMAL	CONTROL SALINE	HISTAMINE, MG./ML. 1.0	0.1	.01
Untreated	37	0	++++	++	+
	38	0	++++		++
Cortisone, 1 Day	32	0	++	+	+
	33	0	0	+	0
	34	0	+	0	0
Cortisone, 2 Days	31	0	0	0	0
	35	0	0	0	0
	36	0	+	0	0

*Degree of response graded according to size and intensity of staining.

TABLE III. EXPERIMENT 5. STAINING RESPONSES OF UNTREATED AND CORTISONE-TREATED RABBITS TO INTRADERMAL HISTAMINE.* TESTED FORTY-FIVE HOURS AFTER LAST OF THREE DAILY INJECTIONS OF 10 MG. CORTISONE

TREATMENT	ANIMAL	CONTROL SALINE	HISTAMINE, MG./ML. 1.0	0.1	.01
Untreated	3	0	++++	+++	++
	8	0	+++	+++	+
	13	0	++++	+++	++
	14	0	+++	++	+
Cortisone	4	0	+++	++	+
	6	0	0	0	+
	7	0	0	0	0
	136	0	0	0	0

*Degree of response graded according to size and intensity of staining.

with and without treatment, each animal served as its own control. Consistent marked inhibition of staining was observed when cortisone was given. Figs. 1 and 2 show typical responses of an untreated and a treated animal.

In Experiment 4 (Table II) cortisone was administered for only one- and two-day periods. One day's administration produced partial inhibition of staining. After two days' administration almost complete inhibition of staining occurred, a result equivalent to that obtained with the four-day treatment.

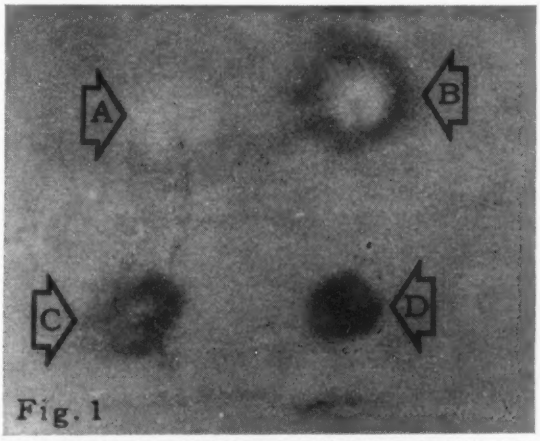

Fig. 1

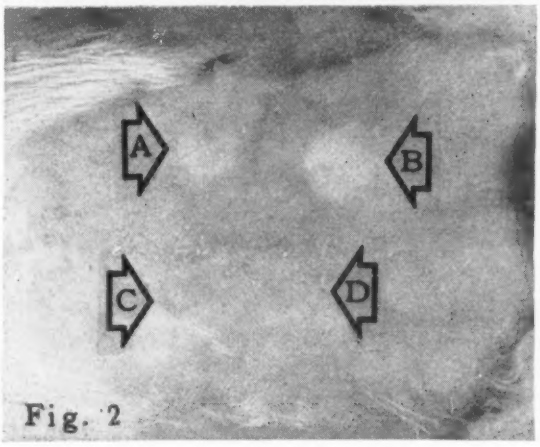

Fig. 2

Figs. 1 and 2.—Typical staining responses, twenty minutes after Evans Blue dye, of an untreated control animal (Fig. 1), and an animal which has received 10 mg. cortisone daily for four days (Fig. 2). Sites of intradermal injections are indicated by arrows. Control saline is at *A*; histamine, 1.0, 0.1, and 0.01 mg. per milliliter are at *B*, *C*, and *D*, respectively. The untreated animal shows good staining at sites of histamine injection. In contrast, the the cortisone-treated animal shows no staining.

In Experiment 5 (Table III) 10 mg. cortisone daily were administered for three days, and the animals were tested approximately forty-five hours after the last injection of cortisone. One animal stained as well as the control animals, but the remaining three showed marked inhibition of staining equivalent to that obtained when testing was done twenty-four hours after the last injection of cortisone.

TABLE IV. EXPERIMENT 6. DYE TRANSFER FROM AN UNTREATED AND A CORTISONE-TREATED ANIMAL (10 MG. CORTISONE DAILY FOR 4 DAYS). DONOR ANIMALS GIVEN TEN TIMES THE USUAL DOSE OF DYE. STAINING RESPONSES OF DONOR AND RECIPIENT ANIMALS*

ANIMAL	TREATMENT	CONTROL SALINE	HISTAMINE, MG./ML. 1.0	0.1	.01
Donors					
4	Untreated	0	++++	++++	++++
136	Cortisone	0	+	0	0
Recipients					
1-x (plasma from donor 4)	Untreated	0	+++	+++	++
4-x (plasma from donor 136)	Untreated	0	++++	+++	++

*Degree of response graded according to size and intensity of staining.

Because cortisone administration to rabbits produces electrolyte changes, hyperlipemia and hyperglycemia,[11] the possibility existed that in treated animals the dye, held in circulation by altered plasma, was unavailable for capillary diffusion. To test this hypothesis, in Experiment 6 (Table IV) first an untreated animal was given 80 mg. of dye. The animal was bled, obtaining 30 ml. of deeply stained plasma calculated to contain a quantity of dye roughly equal to that used in previous experiments. This plasma was administered to an untreated animal on which intradermal histamine injections had been made. The result was good staining at injection sites, demonstrating successful dye transfer.

The above procedure was repeated, giving the large dose of dye to an animal treated with 10 mg. cortisone daily for four days. Thirty milliliters of plasma containing dye was administered to an untreated animal on which histamine injections had been made. The result was good staining, if anything somewhat better than that obtained when the untreated animal was used as a donor of dye-containing plasma.

In connection with this experiment the histamine response of the donor animals, which received ten times the dose of dye used in previous experiments, was also tested. The untreated animal showed intense staining, but the cortisone-treated animal showed only a very light halo at the highest concentration of histamine. Marked inhibition of staining occurred even with such a large dose of the dye.

DISCUSSION

The results demonstrate that cortisone administration to rabbits almost completely inhibits the deposition of Evans Blue dye at sites of histamine injection. The staining observed in an untreated animal which has received dye in the plasma of a cortisone-treated animal is evidence the effect is not due to plasma changes. The marked inhibition of staining occurring even when ten times the usual dose of dye was employed is further evidence against retention of dye by plasma. There appears to be an effect on the local vascular response, presumably due to inhibition of the permeability increase normally induced by histamine.

SUMMARY AND CONCLUSIONS

Cortisone administration to rabbits produces marked inhibition of the cutaneous histamine response, as measured by diffusion of Evans Blue dye.

Evidence is presented that this effect is due to action affecting capillary permeability rather than to retention of dye by altered serum.

REFERENCES

1. Marmorstan-Gottesman, J., and Perla, D.: Effect of Injections of Cortin on Resistance of Suprarenalectomized Rats to Large Amounts of Histamine, Proc. Soc. Exper. Biol. & Med. **28**: 1022, 1931.
2. Hechter, O.: Effect of Histamine upon Capillary Permeability in the Skin and Muscle of Normal and Adrenalectomized Rats, Endocrinol. **32**: 135, 1943.
3. Long, J. B., and Favour, C. B.: The Ability of ACTH and Cortisone to Alter Delayed Type Bacterial Hypersensitivity, Bull. Johns Hopkins Hosp. **87**: 186, 1950.
4. Kass, E. H., and Finland, N.: The Role of Adrenal Steroids in Infection and Immunity, New England J. Med. **244**: 464, 1951.
5. Sprague, R. G.: Cortisone and ACTH. A Review of Certain Physiologic Effects and Their Clinical Implications, Am. J. Med. **10**: 567, 1951.
6. Ebert, R. H. and Wissler, R. W.: In Vivo Observations of the Effects of Cortisone on the Vascular Reaction to Large Doses of Horse Serum Using the Rabbit Ear Chamber Technique, J. LAB. & CLIN. MED. **38**: 497, 1951.
7. Menkin, V.: Further Studies on Effect of Adrenal Cortex Extract and of Various Steroids on Capillary Permeability, Proc. Soc. Exper. Biol. & Med. **51**: 39, 1942.
8. Shleser, I. H., and Freed, S. C.: The Effect of Peptone on Capillary Permeability and Its Neutralization by Adrenal Cortical Extract, Am. J. Physiol. **137**: 426, 1942.
9. Benditt, E. P., Schiller, S., Wong, H., and Dorfman, A.: Influence of ACTH and Cortisone upon Alteration in Capillary Permeability Induced by Hyaluronidase in Rats, Proc. Soc. Exper. Biol. & Med. **75**: 782, 1950.
10. Graham, J. S.: Effect of Carbon Arch Irradiation and Adrenal Cortical Preparations on Capillary Permeability, Proc. Soc. Exper. Biol. & Med. **54**: 101, 1943.
11. Kobernick, S. D., and More, R. H.: Diabetic State with Lipaemia and Hydropic Changes in the Pancreas Produced in Rabbits by Cortisone, Proc. Soc. Exper. Biol. & Med. **74**: 602, 1950.

EFFECTS AND CLINICAL SIGNIFICANCE OF BACTERIAL CONTAMINANTS IN TRANSFUSED BLOOD

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CERTAIN bacteria generally considered to possess little or no pathogenicity may sometimes cause great harm. A striking example is the disturbance seen in recipients of blood containing large numbers of bacterial contaminants. Borden and Hall[1] have recently described in detail two fatal reactions resulting from the transfusion of blood containing massive numbers of bacteria. They reviewed in their report eight additional reactions of this type including those contained in a personal communication from Whitby, who told of one nonfatal and six fatal accidents. Involved in all ten reactions were gram-negative bacilli identified as *Escherichia coli, Pseudomonas aeruginosa, Aerobacter aerogenes, Achromobacter,* and *Paracolobactrum aerogenoides.* A gram-positive organism was isolated only once. The chief clinical characteristic in the ten reactions was peripheral vascular collapse.

The fatal cases represent the ultimate effects of bacterial contamination of transfused blood. Their occurrence, though rare, indicates that less profound clinical disturbances have also resulted, but there are no reports which disclose whether less serious transfusion reactions have been caused by gross bacterial contamination. The entrance of bacteria into some bottles of stored blood appears to be inevitable.[2-5] These facts have led us to study the problem in an attempt to define not only the actual and potential clinical problem but also the basic mechanisms which govern the harmful effects of bacterial contaminants in banked blood.

METHODS

Techniques Used to Obtain Blood From Donors in Blood Bank at University of Michigan.—Two types of bottles were used, both of which were manufactured by the Abbott Laboratories. One of these contained a vacuum and both were supplied with 120 ml. of standard ACD solution composed of 1.32 Gm. sodium citrate, 0.48 Gm. citric acid, and 1.47 Gm. dextrose per milliliter. The donor's skin was anesthetized with 1.5 per cent procaine solution and was cleansed with 70 per cent alcohol before treatment with tincture of iodine. Disposable plastic tubing was connected to bottles of both types by a needle. The citrate was flushed through the tubing attached to the bottle without vacuum and through the vein needle before injecting into the vein. After the venipuncture, the cap of the bottle without the vacuum was also punctured with an autoclaved 15-gauge needle which served as an air vent. When the donation of blood was completed, the needle serving as the air vent was removed. Then the bottle was disconnected from the tubing before removal of the needle from the vein.

Demonstration of Bacteria in Banked Blood.—Cultures were made from blood which had been drawn twenty-four hours previously and stored at 4° C. Smears of these bloods were stained by Gram's method and examined microscopically. A total of 1,697 consecutive

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bottles of blood were examined by removing 3.0 ml. of blood under the strictest aseptic conditions. Two milliliters of blood were inoculated into thioglycollate broth, and 1.0 ml. poured into 9.0 ml. of melted agar. After incubation at 37° C. for twenty-four hours, the broth was examined for bacterial growth, the poured plates for total number of bacterial colonies, and the organisms were identified. Of the 1,697 bloods cultured, 1,192 were collected by the "gravity" method and 505 by the "vacuum" method. In addition, blood was obtained for culture from 200 bottles while the transfusions were in progress.

CLINICAL STUDIES

An attempt was made to record temperature, pulse, blood pressure, and general condition of the recipients at frequent intervals both during and after the transfusion. Unless otherwise stated under "Results", it was felt that the clinical effects of a transfusion were properly observed. If reactions occurred, the transfused blood was again examined for bacterial contamination, compatibility of type, and hemolysis. The level of hemoglobin in the plasma was determined by the method of Flink and Watson.[6]

Search for Source of Contamination.—All needles used in the gravity procedure were cultured by dropping them into a tube of thioglycollate broth and incubating at 37° C. for five days. Growth was checked at the end of that time by subculturing on blood agar if no visible growth had developed in the broth. ACD solution was removed from the bottle with an autoclaved syringe and needle and cultured by injecting 2 to 3 ml. into thioglycollate broth. The tubing was cultured by flushing it through with ACD solution from the bottle and then adding the ACD solution to the broth as described. Cotton swabs used for applying tincture of iodine, gauze pads soaked in seventy per cent alcohol, and a few drops of procaine solution were each dropped into thioglycollate broth. For culturing air in the donor-room, 50 ml. of air were sucked into a sterilized syringe and then bubbled directly into the broth.

Bactericidal Tests of Banked Blood.—A standard bactericidal test was performed by inoculating 0.8 ml. of a saline suspension of bacteria into 4.0 ml. of citrated banked blood. The bottles from which the blood was removed had been refrigerated at 4° C. for twenty-four hours after drawing. The object was to obtain a final density of 200 bacteria or less per milliliter of blood, as this number was in the range of concentration of bacteria generally observed in blood found to be contaminated. Suspensions in saline were made having an initial density approximately equal to the McFarland nephelometric reading of barium sulfate No. 1. This was done by adding 0.2 to 0.6 ml. of a culture, grown for twenty-four hours in tryptose-phosphate broth, to 12 to 15 ml. of normal saline solution. Dilution to 10^{-11} usually provided the desired concentration after addition to the blood, but with some organisms, especially certain strains of *A. aerogenes*, higher dilutions were necessary. Procedures which involved the suspension of bacteria in saline were completed in a few minutes because it was found that for certain bacteria, especially *E. coli*, saline became bactericidal after several hours.[7] The bacterial populations of the inoculated blood were determined after incubation at room temperature by removing 0.5 ml. of blood at various intervals up to twenty-four hours and by making poured plates in melted agar. The colonies were counted after twenty-four hours' incubation of the plate at 37° C. Detailed bactericidal or growth curves were plotted by counting colonies at intervals of 0, ¼, 1, 2, 4, 8, and 24 hours. For testing large numbers of bacteria and bloods, less detailed studies were based on determinations made at 0, 6, and 24 hours. These experiments were controlled by inoculating the organisms into broth instead of blood and plotting the growth. The bactericidal actions of 162 bloods and the survival of 93 strains of bacteria were studied. In addition, a prozone was sought in the bactericidal test by comparing simultaneously the action of blood against four serial dilutions of a saline suspension made from each of fourteen strains of staphylococci. Each strain so tested was exposed to samples of blood from two different donors.

The activity of complement (C′) in the bactericidal or suppressive power of banked blood against contaminating bacteria was determined by performing the standard bac-

tericidal test using citrated plasmas inactivated by heat and NH_4OH. Five strains of coagulase negative staphylococci were tested against three plasmas, three strains of *E. coli* (''L', ''3'', ''100'') against one plasma, and one strain of *E. coli* (''K'') against seven additional plasmas. Each of these plasmas had been heated to 56° C. for twenty minutes and their effectiveness compared to the same plasmas unheated. An attempt was made to restore bactericidal activity to the six heated plasmas tested against *E. coli* ''*K*'' by the addition to each plasma of 0.1 ml. restored undiluted guinea pig serum (Lyo-complement, Sharp and Dohme) containing marked excess of activity as determined in hemolytic systems in this laboratory. The three strains of *E. coli* ''L'', ''3'', and ''100'' were each exposed in the bactericidal test to 4.0 ml. of plasma which had been previously incubated with 1.0 ml. 0.15 NH_4OH and then diluted with 16 ml. of saline. The bactericidal test using plasma treated with NH_4OH to destroy C'4 was controlled by simultaneous tests using untreated plasma, plasma heated to 56° C. for twenty minutes, and plasma diluted from 4 ml. to 22 ml. with saline.

Survival and Growth of Bacteria in Refrigerated Blood Including Studies on Duration of Bactericidal Power.—Samples of blood were inoculated with a bacterial suspension of each of eight strains of *E. coli*, four strains of enterococcus, and six strains of *A. aerogenes*. The inoculations were made as in the bactericidal test except that larger amounts of blood were used to permit repeated tests of the bacterial population during refrigeration. After removal of 1.5 ml. of blood for standard bactericidal tests, the remainder of the inoculated blood was refrigerated at 4° C. At intervals of three to six days, 0.5 ml. of blood was removed and the bacterial population measured over a period of thirty-six days of refrigeration. In the case of *A. aerogenes* and the enterococci, a determination was also made after seventy-one days of refrigeration.

To determine the duration of bactericidal or suppressive power of refrigerated blood, each of 26 samples of blood was inoculated with suspensions containing one of two strains designated in this laboratory as *E. coli K* and *E. coli L*. In these experiments 4.0 ml. of the bacterial suspensions were added to 20.0 ml. of banked blood. The bactericidal power was determined with 1.5 ml. of blood before refrigeration, and then repeated at weekly intervals. Standard bactericidal tests were also performed with seven uninoculated refrigerated bloods by removing 4.0 ml. at weekly intervals and inoculating them with 0.8 ml. suspensions of seven strains of *E. coli* known to be killed by their respective bloods at room temperature.

Study in Vitro of Bacterial Growth in Blood.—Tests were made for hemolysis, change in blood type, and increase in erythrocyte fragility after inoculating 1.0 ml. of recently drawn banked blood with 0.1 ml. of a bacterial culture grown for twenty-four hours in tryptose-phosphate broth. Bloods were not examined if they were clotted or if heavy bacterial growth was not present. Uninoculated bloods were used as controls in every determination. Hemolysis was estimated after forty-eight to one hundred twenty hours according to the method of Flink and Watson[6] for measuring levels of hemoglobin in the plasma. Tests for fragility of erythrocytes were made by the method of Sanford[8] only if hemolysis had not occurred and only in blood manifesting heavy bacterial growth during a period of twenty-four to seventy-two hours after inoculation. Fragility was considered increased if the tonicity of salt solution was greater than that of the control by at least 0.4 per cent in the tubes containing complete and those containing beginning hemolysis. Erythrocytes were typed by slide agglutination before inoculation and after thirty-six to one hundred fourty-four hours' exposure to bacterial growth of seventy-three strains in 113 blood samples. Typing sera were obtained from the American Hospital Supply Corporation and had been checked for potency. One drop of whole blood and one drop of *A* or *B* serum were mixed on a glass slide and then agitated for two minutes at 40° C. After observation for macroscopic agglutination the suspension of erythrocytes was mixed with a drop of saline and then examined under the microscope to avoid confusion by pseudoagglutination. Contaminated cells and plasmas were crossmatched against uncontaminated homologous cells or plasmas. The following bacteria were tested for their effect on type, and the number of strains used is

given in parentheses: *Alkaligenes fecalis* (2), staphylococcus (18), *A. cloacae* (2), *E. coli* (7), *Ps. aeruginosa* (13), enterococcus (11), *A. aerogenes* (12), *B. subtilis* (1), *Paracolon species* (1), *E. freundii* (3), diphtheroids (2), and *K. pneumoniae* (1).

Transfusing Contaminated Blood in Animals.—White male rabbits weighing about 2 kilograms were divided into eight groups, each containing three rabbits, except for one group which contained two rabbits. In each group of rabbits one was transfused with homologous blood heavily inoculated with bacteria at the time of the transfusion, a second with homologous blood which had supported growth of the same bacteria for eighteen hours, and a third with a saline suspension of that strain of bacteria. Each of three strains of staphylococcus (coagulase negative), four strains of *A. aerogenes*, and two strains of diphtheroids was administered to three rabbits in the manner described. This experimental arrangement was designed to distinguish the effects of transfused bacterial contaminants in rabbits due to bacteria alone, from those due to bacteria plus fresh blood, and those due to the products of bacterial growth in blood.

Rabbits were conditioned for the study by measuring rectal temperatures repeatedly during the week preceding transfusion. They were selected for transfusion only if the rectal temperature did not fluctuate more than 0.6° F. during the ninety minutes immediately preceding the test and if, during the week of observation, they had lost no weight, and the rectal temperatures had not exceeded 103.6° F. In those experiments involving transfusion with contaminated blood, each rabbit received his own blood drawn forty-eight hours previously by cardiac puncture and placed in sterile nonpyrogenic ACD solution in concentrations identical to those used in banked human blood. It had been previously determined that transfusion of sterile blood itself would not produce fever. The citrated blood was "banked" at 4° C. Needles, syringes, and all glass containers were prepared to insure freedom from pyrogens by thorough cleaning and heating to 170° C. for two hours in an oven. In all experiments, random samples of solutions were checked for pyrogenicity according to the standards of the U. S. Pharmacopeia.

The bacteria were grown for twenty-four hours in tryptose-phosphate broth from which they were centrifugated, washed three times in normal saline, and suspended in saline so that the density was equal to the number one tube of the McFarland nephelometer. One milliliter of this suspension was added to 10 ml. of "banked" rabbit blood eighteen hours before the transfusion. In addition, one milliliter of the bacterial suspension was added to each of two tubes containing 10 ml. of tryptose-phosphate broth. The blood and broth were incubated at 37° C., and immediately before transfusion the bacteria were again removed from the broth by centrifugation and washed three times. The entire growth from the first broth culture was added to 10 ml. of sterile rabbit blood which had been incubated eighteen hours at 37° C. and the entire growth from the second culture added to 10 ml. of sterile saline. It will be noted that each of the three suspensions (bacteria in freshly inoculated blood, in saline, and in blood inoculated eighteen hours previously) contained bacteria of the same age of growth, and that the final concentration in each was approximately 10^{12} per milliliter as determined by plate counts immediately before injection.

Each rabbit was transfused through the marginal vein of the ear with 7.0 ml. of the suspension of bacteria in blood or saline during a period of two to three minutes. In a rabbit weighing two kilograms, 7.0 ml. corresponds to 250 ml. in a seventy kilogram man. Rectal temperatures were taken with a standard clinical thermometer on three occasions at least one and one-half hours preceding the transfusion, and their mean value was used as the baseline temperature. Rectal temperatures were taken hourly for six hours, every two hours for the next eight hours, and every four hours for the next twenty-four hours. Animals which did not succumb at once were permitted to survive for seven days. Blood for bacterial plate counts and plasma hemoglobin levels was obtained from the central artery of the ear of each rabbit two to three hours after transfusion. The condition of all rabbits was observed and recorded throughout the period of study. Necropsy was carried out immediately after death. Cultures were made of blood from the heart and from

sections of liver, spleen, kidney, celiac nodes, and all lesions. Sections of liver, spleen, kidney, and any abnormally appearing tissues were stained by hematoxylin and eosin and examined microscopically.

RESULTS

Incidence of Contamination of Banked Blood.—Bacteria were cultured from thirty-eight (2.24 per cent) of the 1,697 bottles of blood examined. Contaminants occurred in twelve of 505 (2.37 per cent) bloods drawn by vacuum and in twenty-six of 1,192 (2.18 per cent) drawn by gravity (Table I). There were fourteen bacteria per milliliter or less, except in the case of one blood so heavily contaminated by diphtheroids that they were too numerous to count in the poured plate. The complete list of bacteria found in blood cultured before leaving the bank is given in Table II. The contaminants isolated most frequently were staphylococci which were cultured from twenty-one bloods, and all but two of the twenty-one strains were coagulase negative. Nine bacterial contaminants, never exceeding three colonies per milliliter, were cultured from 200 bloods obtained from the bottle during transfusion (4.5 per cent). There were five coagulase negative staphylococci, two diphtheroids, and two *B. subtilis*.

The major source of contamination in the donor-room was the atmosphere. Twenty of 137 samples of air contained bacteria, principally coagulase negative staphylococci. Diphtheroids and *B. subtilis* were isolated once each from 100 samples of ACD solution, 100 tubings, twenty-four gauze pads soaked in alcohol, and twenty-two procaine needles. One procaine needle was also contaminated with *A. aerogenes*. No other gram-negative bacteria were found.

Clinical Observations.—One clinical reaction resulted from the use of contaminated blood. This was seen in a child transfused with the blood containing

TABLE I. INCIDENCE OF BACTERIAL CONTAMINATION OF BANKED BLOOD

SYSTEM FOR DRAWING BLOOD	CULTURED	CONTAMINATED	
		NO.	PER CENT
Gravity	1,192	26	2.18
Vacuum	505	12	2.37
Total	1,697	38	2.24

TABLE II. BACTERIA ISOLATED FROM THIRTY-EIGHT CONTAMINATED BLOODS CULTURED BEFORE LEAVING THE BANK

	NUMBER OF BLOODS CONTAMINATED
Staphylococcus	21
coagulase +	2
coagulase -	19
Micr. tetragenes	4
B. alkaligenes	3
Diphtheroids sp.	3
Str. viridans	2
B. subtilis	2
Paracolon sp.	1
Enterococcus	1
Ps. aeruginosa	1
E. freundii	1
Total	39*

*One blood was contaminated with *B. alkaligenes* and *Micr. tetragenes*.

innumerable diphtheroids. Of the thirty-eight contaminated bloods discovered in the study, two bottles were not used clinically and six could not be evaluated because they were transfused into patients undergoing extensive surgical procedures. Of the remaining bloods found to be contaminated in the bank, twenty-eight were transfused without producing clinically detectable reactions. One additional reaction occurred but the cause could not be determined. The recipient experienced a chill and the temperature rose 2.8° F. above normal five hours after the transfusion was started. Because these symptoms became apparent after completion of the transfusion, there was no blood for bacteriologic study. An enterococcus had been cultured four days before removal from the bank.

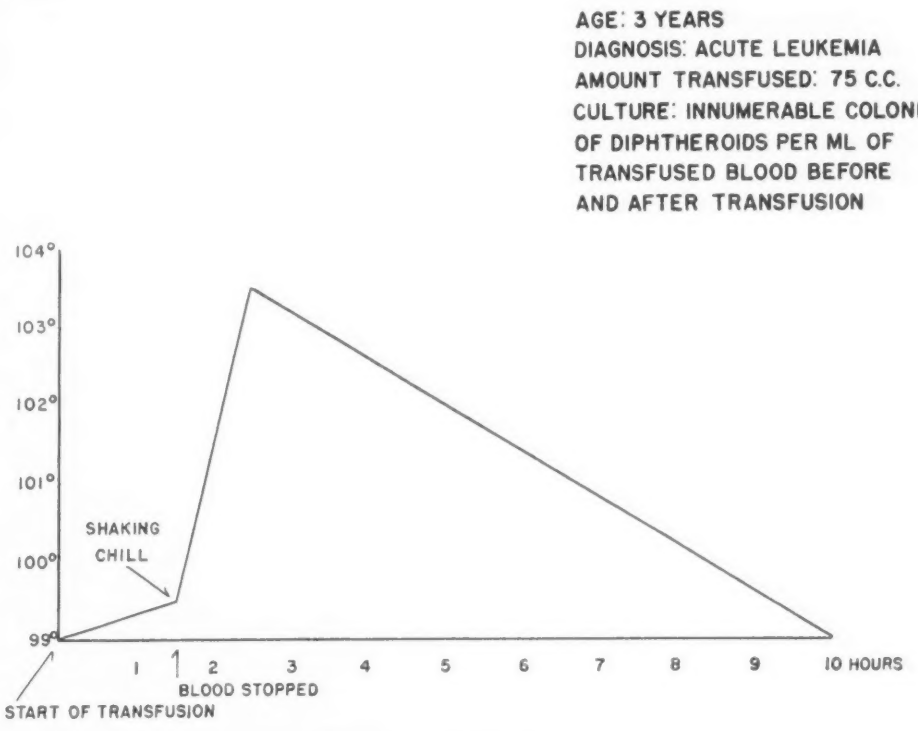

Fig. 1.—Bacterial transfusion reaction due to diphtheroids.

The clinical features of the reaction due to diphtheroids are graphically represented in Fig. 1. The patient was a 3-year-old child suffering from acute leukemia. She had been afebrile before the transfusion. The contaminated blood was transfused immediately after a sample had been removed for bacteriologic examination in the bank so that the results of culture were not then known. A shaking chill was experienced ninety minutes after the start of the transfusion when 75 ml. of blood had gone into the vein. At this point the transfusion was discontinued. The temperature rose abruptly to 103.6° F.

and then fell gradually to normal in ten hours. No other ill effects were observed and the patient left the hospital shortly afterward in good condition. A sample of the blood which remained after the transfusion was stopped was found to contain innumerable diphtheroids as determined by direct smear and culture. To learn if the diphtheroids recovered before and after transfusion were the same organisms, their sensitivity to antibiotics was determined. The diphtheroids isolated in each instance were inhibited by 3.2 μg per milliliter of aureomycin or chloramphenicol, and were resistant to 25.6 μg per milliliter of streptomycin.

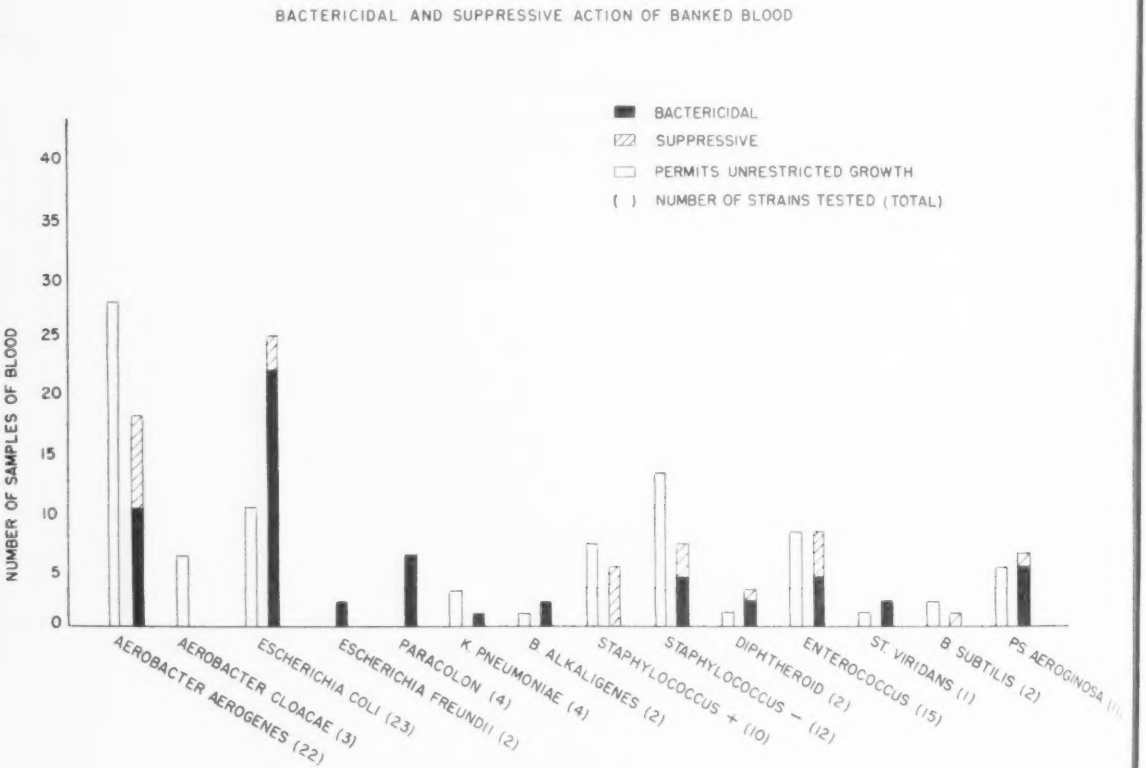

Fig. 2.—Bactericidal and suppressive action of banked blood.

Bactericidal Properties of Blood.—At room temperature, eighty-four bloods killed or suppressed the bacteria with which they had been inoculated and eighty-three bloods permitted heavy growth. Fig. 2 presents a summary of the bactericidal action of these 167 bloods against ninety-three strains. With the exception of *A. cloacae*, among every species tested there were strains which did not grow or were killed by human blood. Bacteria were considered to be killed if their population after twenty-four hours in blood was reduced, even if sterilization had not occurred. Strains of *E. coli* grew in the presence of ten of thirty-five bloods (30 per cent), whereas twenty-eight of forty-six (60 per cent) bloods permitted heavy growth of *A. aerogenes*. Heavy growth of

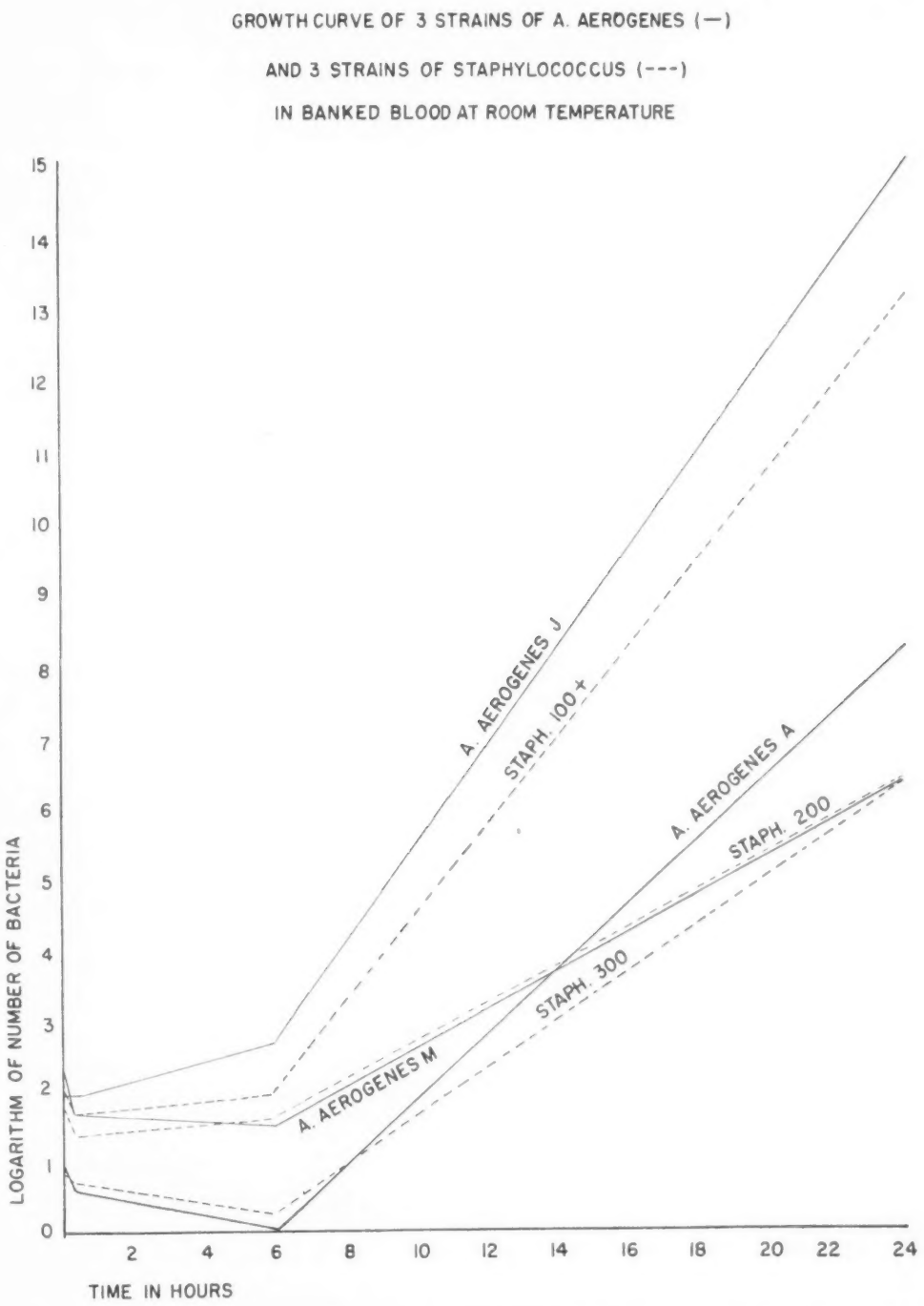
GROWTH CURVE OF 3 STRAINS OF A. AEROGENES (—)
AND 3 STRAINS OF STAPHYLOCOCCUS (---)
IN BANKED BLOOD AT ROOM TEMPERATURE
LOGARITHM OF NUMBER OF BACTERIA
TIME IN HOURS
A. AEROGENES J
STAPH. 100 +
A. AEROGENES A
STAPH. 200
STAPH. 300
A. AEROGENES M

Fig. 3.—Growth curve of three strains of *A. aerogenes* and three strains of staphylococcus in banked blood at room temperature.

staphylococci was observed in eighteen of thirty-one bloods (58 per cent). Four bloods killed coagulase negative staphylococci but coagulase positive strains were never killed. In general, gram-positive bacteria were somewhat more resistant to killing or suppression by blood than gram-negative bacteria. Thus, heavy growth of gram-positive bacteria was seen in thirty of fifty-seven bloods (53 per cent), and of gram-negative bacteria in fifty-two of 110 (47 per cent). Twelve strains of various bacteria, which were killed upon one exposure to blood, manifested heavy growth upon inoculation into blood from a second donor. A zone phenomenon was observed twice among fourteen strains of staphylococci. In one blood the organism grew readily in dilutions of 10^{-5}, 10^{-7}, and 10^{-8}, but were killed in the 10^{-6} dilution. In the other blood, there was suppression at 10^{-6} and 10^{-8}, but growth at 10^{-5} and 10^{-7}.

In all eighty-three bloods which allowed heavy growth in twenty-four hours, the lag phase preceding multiplication exceeded six hours. A lag phase greater than six hours was also observed in broth with inoculums containing the same number of bacteria. Detailed growth curves in blood for three representative strains of staphylococci and *A. aerogenes* are shown in Fig. 3.

In blood stored at 4° C., growth occurred among four strains of *E. coli*, three strains of enterococci, and one strain of *A. aerogenes*. Table III discloses that these bacteria multiplied in the refrigerator only if bactericidal or suppressive power of the inoculated blood had been absent initially or lost after storage. When killing or suppressive power was lost, growth occurred within one week. One strain, *E. coli P*, multiplied until the third week and then disappeared by the fifth. The only strain of enterococcus which was inoculated into blood possessing suppressive action failed to grow significantly in the refrigerator and ultimately died. The populations of two strains of *A. aerogenes*

TABLE III. RATES OF GROWTH OF BACTERIA INOCULATED INTO BANKED BLOOD AND STORED AT 4° C.

STRAIN	NUMBER OF VIABLE BACTERIA PER MILLILITER REFRIGERATED BLOOD DURATION OF REFRIGERATION IN WEEKS								BACTERICIDAL OR SUPPRESSIVE ACTION OF INOCULATED BLOOD
	0	1	2	3	4	5	6	10	
E. coli "K"	1	2	0	0	48	22			Bactericidal action lost after third week
E. coli "G"	21	14	I*	I*	I*				Suppressive action lost after first week
E. coli "P"	0	1	2	I*	100	0			Suppressive action lost after second week
E. coli "E"	7	171							None present initially
Enterococcus "1"	52	65	145	175	314	536	805	I*	None present initially
Enterococcus "6"	60	113	134	106	218	611	810	I*	None present initially
Enterococcus "9"	88	73	166	431	1,061	I*	I*	I*	None present initially
A. aerogenes A	24	50	48	138	70	115		I*	None present initially

*Innumerable.

and four of *E. coli* inoculated into bactericidal bloods were markedly reduced within a short time after refrigeration. Three strains of *A. aerogenes* survived in refrigerated blood not possessing bactericidal or suppressive power, and their number gradually diminished after three weeks.

Loss of bactericidal power occurred at 4° C. among four of twenty-six bloods inoculated with *E. coli "K"* or *E. coli "L"*. In each case this occurred after two weeks of refrigeration. The remaining twenty-two bloods retained bactericidal power for four weeks or longer. Of the seven refrigerated bloods inoculated weekly with freshly cultured strains of *E. coli*, three lost bactericidal power after one week, one after three weeks, while three bloods remained actively bactericidal up to five weeks.

TABLE IV. LEVELS OF HEMOGLOBIN IN BANKED BLOOD AFTER BACTERIAL GROWTH

NO. STRAINS TESTED	SPECIES	NO. OF HEMOLYTIC STRAINS	LEVELS OF HEMOGLOBIN IN PLASMA (MG. %)
7	*A. aerogenes*	2	A. 2,665 B. 137
3	*E. freundii*	1	1,115
2	Paracolon	2	A. 1,400 B. 1,345
1	*B. subtilis*	1	1,350
4	*E. coli*	1	477
1	*B. alkaligenes*	1	107
2	Enterococcus	0	
8	*Staph. aureus*	0	
1	*A. cloacae*	0	
1	*Ps. aeruginosa*	0	

TABLE V. EFFECT OF NONHEMOLYTIC BACTERIA ON FRAGILITY OF ERYTHROCYTES IN BANKED BLOOD

SPECIES	NO. OF STRAINS	NO. OF BLOODS	STRAINS PRODUCING INC. MECH. FRAGILITY	STRAINS PRODUCING INC. OSMOTIC FRAG.
Enterococcus	3	3	1	3
K. pneumoniae	2	3	0	2
A. aerogenes	3	3	0	1
Ps. aeruginosa	2	2	0	0
Staph. aureus	10	15	0	2
Total	20	26	1	8

Heating to 56° C. for twenty minutes destroyed the bactericidal power of seven plasmas for *E. coli* but not that of three plasmas for coagulase negative staphylococci. Addition of C′ from guinea pigs did not restore the lost killing power. Plasma treated with .015N NH_4OH lost its killing power for the three strains of *E. coli* tested.

Effect in Vitro of Bacterial Growth in Blood.—Levels of hemoglobin in the plasma higher than 100 mg. per cent were caused by eight of thirty-one strains (Table IV). Eight nonhemolytic strains distinctly increased erythrocyte fragility (Table V). Among fourteen species, there were 125 strains which clotted blood by consuming citrate, a phenomenon considered in detail in another report.[9] The blood type was altered only once in 113 contaminated specimens

when type O was changed to AB by *Ps. aeruginosa*. Clumping also occurred when the cells and plasmas contaminated by this strain of *Ps. aeruginosa* were crossmatched against uncontaminated homologous cells and plasmas. Another strain of *Ps. aeruginosa* did not alter the erythrocytes but changed the sera from a type O specimen so that homologous cells were clumped. Neither strain of pseudomonas altered blood on further testing.

Effects in Animals Transfused with Contaminated Blood.—Rabbits transfused with blood or saline containing large numbers of staphylococci or diphtheroids experienced fever but no other observable harm. By comparison, *A. aerogenes* also produced fever but, in addition, all animals inoculated became ill and all but one died (Table VI). The sickest animals suffered in turn diarrhea, dyspnea, extreme weakness with inability to stand, generalized clonus, opisthotonos, complete flaccid paralysis, and death. The first symptom was noticed in one hour, and death occurred as early as two and one-half hours. Some animals manifested only lethargy until shortly before death. The three rabbits, transfused with blood inoculated with *A. aerogenes* eighteen hours previously, died before nine hours had elapsed. All other rabbits receiving suspensions of *A. aerogenes* in blood or saline, survived for more than nine hours except one which lived only two and one-half hours after transfusion of a bacterial suspension in saline. Two rabbits survived seven days. One died at that time and the other, which had become greatly emaciated and prostrate, was sacrificed. No hemolysis had occurred prior to transfusion in the bloods inoculated eighteen hours before use regardless of species used. Intravascular hemolysis was observed in only two rabbits, both of which had received the same strain of *A. aerogenes*. This organism had been isolated originally from the blood of a septicemic patient who had experienced marked intravascular hemolysis. In one of the rabbits, the plasma hemoglobin level became elevated to seventy-five milligrams per cent three and one-half hours after receiving a saline suspension of this culture. The other rabbit received blood inoculated eighteen hours previously with this organism and his plasma hemoglobin rose to thirty-five milligrams per cent in three and one-half hours.

Rabbits appeared unable to clear *A. aerogenes* from the blood as rapidly as staphylococci or diphtheroids. In two to three hours there were 0 to 50 diphtheroids and 12 to 20 staphylococci per milliliter of blood in all except one rabbit whose blood contained 1,000 staphylococci per milliliter. The numbers of *A. aerogenes* in the blood after two to three hours, however, were often too numerous to count in the regular plate poured with 0.5 ml. of blood, and

TABLE VI. EFFECTS OF TRANSFUSING RABBITS WITH BLOOD OR SALINE CONTAINING 10^{12} BACTERIA PER MILLILITER

SPECIES	NO. OF STRAINS	MEAN FEBRILE RESPONSE	TOXICITY	MORTALITY RATE
A. aerogenes	4	3.5 degrees in 3.16 hours	+ + + +	11/12
Staphylococcus	3	4 degrees in 4.5 hours	0	0/8
Diphtheroids	2	2.8 degrees in 3.2 hours	0	0/6

generally exceeded 100 per ml. Culture of tissues obtained post mortem from such animals disclosed innumerable colonies of *A. aerogenes* in the liver and spleen. In the animals sacrificed after one week, culture of the liver yielded no *A. aerogenes*, but numerous colonies were recovered from the spleen. Cultures of the tissues of animals who had received diphtheroids were sterile at the end of one week while culture of the liver and spleen of rabbits sacrificed one week after transfusion with staphylococci showed only an occasional colony of this organism.

There were no gross anatomic changes observed post mortem. Microscopic examination of the spleen and sometimes the liver of animals transfused with blood containing *A. aerogenes* disclosed marked focal necrosis. In the spleen the necrosis was sometimes very extensive, involving all elements of the pulp and encroaching upon the follicles. Masses of bacteria were observed in the necrotic centers. The tissues examined from rabbits inoculated with staphylococci and diphtheroids were not found to be abnormal.

DISCUSSION

The true incidence of contamination of banked blood existing during the period of these studies was probably higher than the observed rate of 2.24 per cent. The large remaining portion of blood used for transfusion undoubtedly contained bacteria not represented in the small aliquot examined. As these observations have been made in a university hospital whose methods are constantly open to scrutiny during the course of teaching, and whose personnel is well selected and trained, the opportunity for contamination of transfused blood may be less than that which exists in hospitals with less supervision. Contamination of some bottles of transfused blood is, therefore, to be expected, and recipients are potentially exposed to the harmful effects disclosed by the present investigation. It appears that the most serious danger is transfusion of blood containing enough gram-negative bacilli to cause the violent toxic action seen experimentally in rabbits and clinically in cases reported elsewhere.[1] There is also potential harm from hemolysis, change in type, and increase in erythrocyte fragility, all resulting from bacterial growth.

Fortunately the actual risk from contamination was found to be much less than the rate of contamination might indicate because of factors which minimize these hazards. The commonest contaminants in the blood and in the donor-room were nonpathogenic staphylococci. These organisms, though pyrogenic, have been demonstrated to elicit no harmful signs of consequence even when huge numbers were given to animals in transfused blood. Diphtheroids appeared harmless, also, both in animals and in the one patient who experienced only a febrile reaction despite the heavy contamination of the transfused blood. It would seem that nonpathogenic gram-positive contaminants may not be harmful in man even after transfusion of blood containing huge numbers of organisms. Thirty-three of thirty-nine contaminated bloods contained gram-positive bacteria, and only six contained gram-negative bacteria.

The small incidence of serious reactions from contaminated blood is partly explained by a study of growth of bacteria in blood and by the bactericidal or

suppressive action of blood. Approximately half of the bacterial contaminants tested were killed or suppressed by banked citrated blood standing at room temperature. Among the fifty per cent which survived in blood and grew heavily in twenty-four hours at room temperature, the lag phase preceding multiplication always exceeded six hours, a period longer than required for giving transfusions. At 4° C., the storage temperature for banked blood, growth appeared to be suppressed in blood which possessed bactericidal or suppressive power. If these antibacterial properties were lost during storage, or if they were not present to begin with, heavy multiplication sometimes developed. In the case of gram-negative bacteria, the bactericidal power of blood was found to depend on complement (C′) as demonstrated by loss of the killing power of plasma for *E. coli* when C′ was inactivated by heat and C′4 by NH_4OH. Failure to restore killing with C′ from guinea pigs was not surprising as their sera are known to be a poor source of C′ for the bactericidal test.[10] Most bloods inoculated with *E. coli* retained bactericidal action for more than four weeks at 4° C. This is in accord with the long duration of activity of C′ in citrated blood at cold temperatures as demonstrated by Kolmer.[11] The occasional loss of bactericidal action which occurred in refrigerated blood was probably due to loss of C′. The preservation of C′ for many weeks in banked blood during refrigeration is no doubt important from the standpoint of protecting recipients from the effects of gram-negative bacterial contaminants.

The factors which minimize bacterial growth in blood protect the patient not only from the direct effects of the bacteria but also from the hemolysis, from the increased fragility, and from the change in type which may result from bacterial action on erythrocytes. The relative infrequency with which staphylococci produce these changes is also significant. Hemolysis and change in blood type by staphylococci were not observed in the present experiments, and increased fragility was produced by only two of ten strains of staphylococci. By contrast, among gram-negative bacteria, marked hemolysis in banked blood resulted from growth of seven of nineteen strains. When eight of the nonhemolytic gram-negative strains were examined further, three were found to increase the fragility of erythrocytes.

Change in blood type by bacteria is uncommon and is, therefore, not a frequent hazard from contamination of the transfused blood itself. A more likely source of difficulty will occur from the contamination of pilot specimens used for typing and crossmatching, because sterile precautions are not universally observed in their collection and storage. Typing may be interfered with by the "H" phenomenon of Davidsohn and Toharsky[12] which involves the production by bacteria in serum of agglutinins for all human cells. Typing may also be disturbed because of the "T" phenomenon of Friedenreich[13] which is characterized by the bacterial transformation of erythrocytes into cells which are agglutinated by all adult human sera. Such altered cells are typed as AB in the routine tests. Each phenomenon was observed in our experiments and was induced by two different strains of *Ps. aeruginosa* but by no other bacteria.

This change in erythrocytes was possibly related to the great proteolytic activity of *Ps. aeruginosa* and can be compared to the change in erythrocytes by trypsin.

Consumption of citrate led to the clotting of blood by fifty-four of eighty-three strains of gram-negative bacteria including sixteen of eighteen strains of *A. aerogenes*.[9] Clotting of banked blood is not a serious clinical hazard because of the filter systems used. The presence of clots, however, may be strongly indicative of heavy bacterial growth, and serves as a useful signal that the banked blood may be contaminated.

The predominant isolation of nonpathogenic staphylococci and other gram-positive bacteria from articles and air in the donor-room explains the frequent contamination of blood by these bacteria. Sampling of the air yielded more frequent positive cultures than from any other source, a fact which emphasizes the importance of preventing entry of air into blood bottles during collection. Less contamination was expected when blood was collected by a "closed" vacuum system, but the incidence of contamination was the same for gravity and vacuum methods. Bacteria were allowed to enter the blood bottle when air was sucked around the outside of the needle attached to the vacuum bottle. Bacteria were isolated twice as often from samples of blood removed from the bottle during transfusion as from specimens obtained twenty-four hours after drawing in the bank. The number of colonies per milliliter of blood had not increased, however, at the time of transfusion. In other words, the process of administering the transfusion led to additional contamination, but growth was restricted by the factors already mentioned.

One may conclude that under the conditions studied, banked blood is not infrequently contaminated, but the number of bacteria per milliliter of blood are few and their threat to the recipient is small. The rare serious reactions from bacteria will occur only when blood becomes so heavily inoculated with gram-negative bacilli that the lag phase is shortened and the bactericidal or suppressive actions overcome. The recipient of blood so contaminated by gram-negative bacilli may face the possible grave effects of hemolyzed blood, of more fragile erythrocytes, and most certainly of bacterial toxins. Blood may also become heavily contaminated and overgrown by gram-positive bacteria, leading to fever but without other apparent harm to the patient. A number of benign reactions undoubtedly result from blood heavily contaminated with nonpathogenic gram-positive bacteria.

SUMMARY AND CONCLUSIONS

Bacterial contaminants were isolated from thirty-eight (2.24 per cent) of 1,697 pints of blood cultured before leaving the bank. Organisms most frequently isolated were nonpathogenic staphylococci.

Heavy growth by diphtheroids produced the only febrile reaction among recipients of these contaminated bloods. The patient experiencing the reaction suffered no observable harm.

The low rate of reaction was explained by (a) the bactericidal or bacteriostatic action of approximately fifty per cent of the bloods, (b) the long lag

phase preceding multiplication of bacteria in blood not possessing bactericidal or suppressive power, (c) infrequent growth of bacteria in the refrigerator, and (d) small size of the bacterial inoculum.

The potential harm from these contaminants was investigated by observing the effects of heavy growth in human blood. Marked hemolysis, increased erythrocyte fragility, and clotting due to consumption of citrate were frequently caused by gram-negative bacteria but seldom by staphylococci. Alteration of erythrocytes leading to change in type was found only once in 113 contaminated specimens.

The potential harm in vivo of heavily contaminated blood was determined by comparing the effects of transfusing blood containing either staphylococci, diphtheroids, or *A. aerogenes* into rabbits. Staphylococci and diphtheroids invariably produced fever but no harm. *A. aerogenes*, on the other hand, was equally pyrogenic but highly toxic and lethal. Necropsy disclosed areas of focal necrosis in the spleen and liver of rabbits killed by *A. aerogenes*.

These harmful effects resulting from transfusion of contaminated blood probably occur clinically only under conditions which permit contamination of banked blood by gram-negative bacteria in numbers large enough to shorten materially the lag phase and to overcome the bactericidal and suppressive actions.

Illustrations were drawn by Joan Tillman.

REFERENCES

1. Borden, C. W., and Hall, W. H.: Fatal Transfusion Reactions from Massive Bacterial Contamination of Blood, New England J. Med. **245**: 760-765, 1951.
2. Swedberg, B., Widstrom, G., and Alin, K.: The Importance of Aseptic Technique and the Fallaciousness of Chemical Bacteriostatics in Blood Banking and Plasma Preserving, Acta Med. Scandinav. **127**: 480-493, 1947.
3. Novak, M.: Preservation of Stored Blood with Sulfanilamide, J. A. M. A. **113**: 2227-2229, 1939.
4. Bick, Marjorie: Some Observations on Certain Bacteriological and Serological Examinations of Stored Blood, M. J. Australia **1**: 265-268, 1945.
5. Elliott, G. A., MacFarland, R. G., and Vaughan, J. M.: The Use of Stored Blood for Transfusion, Lancet 384, 1939.
6. Flink, E. B., and Watson, C. J.: Method for Quantitative Determination of Hemoglobin and Heme Pigments in Feces, Urine, and Blood Plasma, J. Biol. Chem. **146**: 171-178, 1942.
7. Braude, A. I., Bartlett, J., and Sanford, J.: Marked Differences in the Survival of *A. aerogenes* and *E. coli* in Normal Saline, to be published.
8. Todd, J. C., and Sanford, A. H.: Clinical Diagnosis by Laboratory Methods, ed. 10, Philadelphia, 1943, W. B. Saunders and Co., p. 237.
9. Braude, A. I., Sanford, J., and Bartlett, J.: Effect of Sodium Monofluoroacetate on Bacterial Metabolism of Citrate in Human Blood, Fed. Proc. **2**: 409, 1952.
10. Irwin, M. R., and Berman, D. T.: Bactericidal Tests in Brucellosis, *Brucellosis:* A Symposium published by the A. A. A. S., Washington, D. C., 85-98, 1950.
11. Kolmer, J. A.: Preserved Citrated Blood "Banks" in Relation to Transfusion in the Treatment of Disease with Special Reference to the Immunologic Aspects, Am. J. M. Sc. **197**: 442-452, 1939.
12. Davidsohn, I., and Toharsky, B.: The Production of Bacteriogenic Henagglutination, J. Infect. Dis. **67**: 25, 1940.
13. Friedenreich, J.: The Thomsen Hemagglutination Phenomenon, Copenhagen, 1930, Levin and Murksgaard.

BRONCHOSPIROMETRY

I. Review of the Literature

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THE separate and simultaneous recording of the function of the two lungs by bronchospirometry was first performed in man by Jacobaeus, Frenckner, and Björkman in 1932.[1] Since then, this technique has grown from a complex research procedure to a clinical method of practical necessity. Despite the rapid growth of interest in the technique and the increasing number of publications concerning particular facets of bronchospirometry, information on the subject is wanting in a number of respects. The work in this field has not been reviewed since 1934.[2] The technique has never been described in great detail. Contraindications have been enumerated, but complications due to the procedure have only been alluded to. Extensive experience with bronchospirometry has been mentioned occasionally; invariably, only a few striking illustrative cases have been shown or the findings in just one particular disease or operation have been analyzed. Statistical examination of results with various catheters, different medications, and recording techniques have not been made. The consistency in obtaining satisfactory tracings, the severity of dyspnea during the procedure, the cause of unsatisfactory tests, and the incidence of complications have not been reported. The present series of papers has been planned to review the literature and to present a number of variations in technique and new applications of bronchospirometry, which we have found useful during our experience with about 1,000 examinations.

The study will be introduced by a review of the literature. Further papers of this series are planned which will deal with a comparison of available bronchospirometric catheters and experimental and theoretical analysis of the effects of resistance breathing.[3] The study will be continued with a presentation of the technique of bronchospirometry, and with the interpretation of the normal, abnormal, and faulty bronchospirogram. A discussion of indications will be followed by enumeration of contraindications and statistical justification for these contraindications will be given.[4] The special techniques of ambient air recording spirometry[5] and differential residual volume determination will be described.[6] Results will be summarized both from the clinical and the physiologic viewpoint, and the outcome of special studies in-

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cluding bronchial obstruction experiments and differential pneumotachography will be reported.[7] The study will be concluded with an account of experiences with bronchospirometry during intrathoracic surgical procedures.[8]

REVIEW OF THE LITERATURE

For convenience and rapid orientation, publications on the general subject of differential lung function have been grouped according to date, either prior to, or after Jacobaeus, Frenckner, and Björkman's original publication of 1932.[1] Experiments aiming at the solution of problems in respiratory physiology have been separated from those devoted primarily to the study of the effect of pulmonary disease and studies of the effect of collapse and thoracic surgical procedures. Finally, publications concerning primarily the development and description of new instruments for bronchospirometry have been treated separately.

Studies of Differential Function Before 1932.—Techniques for separate air collection from the two lungs, prior to the introduction of the double lumen bronchoscope, have been exhaustively reviewed by Björkman.[2] All early animal studies involved the use of a "Pflüger" catheter, a thin tube with an inflatable rubber cuff at the bronchial end, usually introduced through a tracheotomy. Early tubes were too small for adequate ventilation and were merely used to obtain gas samples. Later, two larger catheters were introduced into the main stem bronchi for study of regulation of respiration, the effects of vagus and sympathetic nerve section, and the unilateral breathing of carbon dioxide and ozone. The consequences of unilateral pneumothorax and of occlusion of one branch of the pulmonary artery were also investigated by these techniques. Because of the defects in the mediastina of the experimental animals used, these studies were not productive until special operative procedures for exposure of the trachea and hilus were developed.[9]

Publications Since 1932.—The double-lumen bronchoscope: Some attempts were made prior to 1932 to measure differential pulmonary function in man,[10, 11] but Jacobaeus, Frenckner, and Björkman were the first to do this successfully.[1] Their original technique was not bronchospirometry in the modern sense. They recorded the respiratory excursions of the two lungs separately, but not simultaneously with a single lumen bronchoscope. Realizing that oxygen uptake and carbon dioxide output change from minute to minute in disturbed patients, they merely recorded ventilation and vital capacity first of one lung and then of the other in eleven patients. The tracings obtained were not strictly comparable because the ventilation varied from one tracing to another. The vocal cords often closed around the tube and obstructed the airway to the lung not under observation.

A double-lumen bronchoscope, consisting of a small round tube within another larger one, was constructed for simultaneous registration of respiratory excursions of the two lungs. Resistance breathing was encountered within the outer tube of semilunar cross section. At the suggestion of Björkman[2] another double lumen bronchoscope was built by Frenckner which consisted of two identical tubes of semicircular cross section placed beside each other.[12] This device merits more detailed description since all but two of the later flexible catheters incorporated its basic design. The single rigid bronchoscopic tube was divided by a partition into two parallel channels of equal and sufficiently large cross section to permit relatively free ventilation of both lungs. Glass windows at the proximal ends of the tubes permitted constant observation of the bronchoscope's position. The slightly longer, protruding tip of the left channel was placed in the left main bronchus, up to, but not beyond the orifice of the upper lobe of the left lung. The left lung was then sealed from the outside by inflation of a rubber cuff proximal to the orifice of the left upper lobe but still within the bronchus. The shorter channel for the right lung ended just above the carina and was sealed by inflation of a larger rubber cuff placed around the entire bronchoscope within the trachea. Björkman described this instrument, the associated recording apparatus, and his experiences with seventy patients in a classical treatise.[2] In spite of the many contributions made by Jacobaeus and his associates using this instrument,[13-16] it did not find general acceptance. This was because the urgent need for clinical bronchospirometry had not arisen and the large stiff broncho-

scope caused considerable discomfort. Although Jacobaeus' publications were reprinted in many countries, we found only a single report of another group using his double bronchoscope.[17]

Analysis of differential function without intubation: A number of techniques have been described for estimation of individual lung function without introduction of instruments into the respiratory passages.

The most widely accepted of these methods is the fluoroscopic observation of the chest during quiet breathing and during maximal ventilatory efforts.[18, 19] This examination has become part of the routine of cardiorespiratory evaluation because it supplies significant information which cannot be obtained by bronchospirometry. Localized "trapping" of air or motion of the mediastinum on forced expiration gives indication of a type of unilateral disease which is missed during bronchospirometry, since the latter of necessity entails some resistance breathing. Fluoroscopy and examination of inspiratory and expiratory films are now used as an adjunct to, rather than a substitute for bronchospirometry. Early split function studies showed that estimation of relative function by roentgenologic means was frequently inaccurate, particularly with respect to oxygen uptake.[1, 2] The discrepancy between functional estimation by roentgenograms and bronchospirograms has been emphasized frequently. Vaccarezza and associates[20] and Johns and Seabury[21] have devoted special reports to an analysis of this subject.

Special grids for registration on one film of both the inspiratory and expiratory position have been developed. Birath and associates have used such doubly exposed roentgenograms for estimation of differential function[22, 23] and Vaccarezza and associates have estimated respiratory excursions of the two lungs in various positions with the help of these "digrafias."[24, 25]

Simonin and associates mentioned a refinement of the roentgenologic technique.[26] The participation in total function of the two lungs was calculated from photoelectrically measured densities of the two lung fields from roentgenograms taken on full inspiration and on deepest expiration. Since the measurement of density is two-dimensional and the expansion of the lungs is three-dimensional, the applicability of this "densidigraphie" must be questioned.

A mechanical device may be mentioned here, the use of which was based on the premise that pulmonary insufficiency is always accompanied by a corresponding limitation of motion of the thoracic cage and the diaphragm. The instrument, described by Labesse, consists of two thoracometers and a device for separate compression of each hemithorax and each leaf of the diaphragm.[27] Ordinary spirometry is performed while the instrument blocks respirations first of one lung and then of the other. Many years ago, similar experiments with double thoracometers led Anthony and Hansen to the conclusion that differential functional analysis in man is impossible.[28]

Occlusion of one branch of the pulmonary artery by an inflatable cuff attached to a cardiac catheter represents perhaps the greatest refinement in differential functional analysis. This technique has been used alone[29] and in conjunction with bronchospirometry.[30] It differs from the previously considered methods because it permits analysis of differential oxygen uptake.

Analysis of differential function by intubation but without bronchospirometry: The original Jacobaeus technique has been modified repeatedly. While most efforts have been directed toward simplification of bronchospirometry, a few investigators have felt that simultaneous recording of respiratory excursions of both lungs is too complex a procedure to be clinically applicable. They returned to Jacobaeus' first method of recording ventilation of each lung in sequence.[1] Bezançon used an ordinary 7-40 Jackson bronchoscope with a rubber cuff at the bronchial end.[31] He recorded respirations for two minutes on each side. The technique was said to be less traumatic and satisfactory for clinical evaluation. Arnaud, Tulou, and Merigot passed a long thin rubber tube, similar to a gastric tube, through the nose into either main bronchus under fluoroscopic control.[32] Respirations were recorded with an ordinary basal metabolism apparatus and mouth piece. After a few minutes the airway to one lung was blocked by inflation of a rubber tampon attached to the tip of the tube. The spirogram was observed for increase in respiratory

rate, decrease in tidal volume and decrease in vital capacity. The procedure was repeated for the other lung. They thought this method avoided the trauma of bronchospirometry and simulated conditions encountered during therapeutic collapse measures or resection. This technique has found some favor on the continent.[33] Subsequently, other investigators have returned to the "Arnaud Test." They used a modern double-lumen catheter and intermittently occluded one or the other lumen.[34, 35] A similar technique, without the refinement of recorded respiratory excursions, was reported in this country by Rogers.[36] Respiratory and pulse rates were noted during bronchoscopy. The main bronchus of the lung to be operated on was then occluded by a sponge attached to a ureteral catheter. The vital rates were then recorded again for fifteeen minutes and observations concerning cyanosis were made. An acceptable candidate for surgery was one who did not develop cyanosis and who showed no appreciable rise of pulse and respiratory rate upon bronchial occlusion.

Modern double-lumen bronchospirometry: The majority of investigators have realized the importance of *simultaneous* recording of respirations from both lungs and have devoted their efforts toward construction of less traumatic and more adaptable double-lumen tubes. Although a new rigid double-bronchoscope has been described recently[37] it is the three flexible double-lumen catheters of Gebauer,[38] Zavod,[39] and Carlens[40] which have brought bronchospirometry within reach of the clinician.

Modifications of the double-lumen bronchoscope: The only stiff double-lumen tube reported since 1934 was designed by Breton and Méréau.[37] It is a complex tube-within-tube arrangement used for functional analysis of single lobes and for unilateral airway obstruction—the "Arnaud test." With it, fifty-five successful examinations were performed; its use has not been recorded by anyone else.

Flexible double-lumen catheters: Three different flexible tubes have been devised to replace the original Jacobaeus double-lumen bronchoscope. With these two distinct advantages of the bronchoscope were sacrificed: the position of the distal ends of the tube within the trachea and left main bronchus could no longer be visualized during bronchospirometry; and the removal of secretions by suction catheter became a great deal more difficult. On the other hand, the flexible tubes introduced numerous advantages. Patients were no longer required to assume the awkward position required for bronchoscopy, but could lie on their backs with a pillow supporting the head. This eliminated the necessity of a head holder, and avoided the pressure against the back of the throat and upper incisors, a common complaint of bronchoscopy. The greater comfort and lesser trauma with the flexible tubes permitted recording of tracings for longer periods of time and patients were willing to submit to the procedure on repeated occasions.

The Gebauer tube: This was the first of the bronchospirometric catheters, described in 1939.[38] It is a double lumen rubber tube with a curvature at the distal end adapted for the take-off of the left main bronchus (Fig. 1). This curvature is supported by a steel spring within the rubber envelope, giving the tube some stiffness which aids in its introduction through the vocal cords and into the bronchus. The spring, by making the bronchial end radiopaque, helps in the fluoroscopic positioning. A single rubber capillary, incorporated in the same rubber envelope, is used to inflate the small rubber cuff within the left main bronchus as well as the larger cuff within the trachea. The left lumen has an end opening for collection of air from the left lung. The right lumen has a lateral opening facing the tracheal wall for collection of air from the right lung. This is an unfortunate defect in design, since this lateral opening tends to become occluded by the wall of the trachea if any abnormal curvative or deviation exists. The significance of this will be discussed in more detail later.

The Gebauer tube was modifiedd by Wright.[41] Both internal diameters were enlarged and the external diameter at the tip was somewhat reduced. Thinner rubber balloons were used for cuffs and additional radioopaque markers were added for fluoroscopic localization of the balloons and the two orifices. It is this modification of the Gebauer catheter which is now commercially available, and which we have used. The catheter is illustrated in Fig. 1 and its dimensions and characteristics are shown in Table I.

The Zavod tube: This catheter was designed independently of Gebauer's and was first described in 1940.[39] It is almost identical in design to the tube just described, but has a flexible steel plate at the bronchial end for maintenance of the natural curvature. Unlike the original Gebauer tube, it has two fine rubber capillaries within its wall for separate inflation of two cuffs at different pressures. It is designed for introduction by indirect laryngoscopy with a steel stylet. It is illustrated in Fig. 1, and the dimensions are summarized in Table I.

The Carlens tube: This, the latest of the bronchospirometric catheters (1949), originated, like the Jacobaeus bronchoscope, in Sweden.[40] It is based on the general principle of the Zavod and Gebauer instruments. However, several changes in design appear

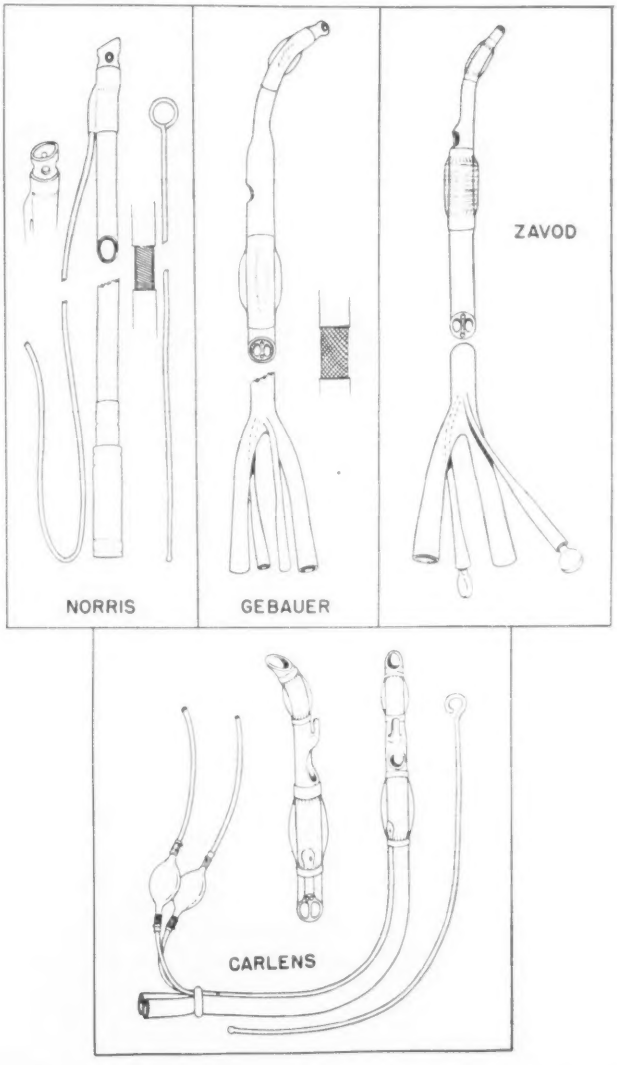

Fig. 1.—Various bronchospirometric catheters. *Above*, from left to right: the single lumen Norris tube with stylet for introduction, the Gebauer catheter as modified by Wright, and the Zavod catheter. *Below*, the Carlens catheter and its stylet. Note the second curvature for the upper respiratory passage, the rubber hook for engagement with the carina, the end rather than side opening facing the right main bronchus, the large internal diameters and the control bubbles for cuff inflation.

TABLE I. COMPARISON OF PHYSICAL CHARACTERISTICS OF BRONCHOSPIROMETRIC CATHETERS

CHARACTERISTICS	GEBAUER TUBE WRIGHT MODIF.		ZAVOD TUBE		NORRIS TUBE		CARLENS TUBE	
	LEFT	RIGHT	LEFT	RIGHT	LEFT	RIGHT	LEFT	RIGHT
Internal diameter, mm.	4-5	5-6	3-5	4-5	7	Variable	7	7
Resistance, cm. of water at 40 L./min. rate of flow[3]	43	28	41	22	8	17-58	7	5
External diameter of tip, mm.	9-11		9-11		10		11	
Orientation of proximal aperture	End	Lateral	End	Lateral	End	?	End	End
Removal of secretions	Difficult	Difficult	Difficult	Difficult	Easy	Impossible	Easy	Easy
Internal lining	Smooth		Smooth		Rough		Smooth	
Maximal external diameter, mm.	12-13		12-13		11		14-15	
Stylet required for introduction	No		Preferably		Yes		Preferably	
Curvature for left bronchus	Yes		Yes		No		Yes	
Curvature for trachea	No		No		No		Yes	
Rubber hook for carina	No		No		No		Yes	
Tubing for cuff inflation	Common or Separate		Separate		One only		Separate	
Control bubbles for cuff inflation	No		No		No		Yes	

to correct some of the defects of the former two instruments. In addition to the curvature at the tip for the left main bronchus, the Carlens tube has a second curvature which corresponds to the position of the trachea and upper respiratory passages of a supine subject whose head is supported on a small pillow (Fig. 1). The instrument is made of thinner rubber, making possible airways of larger internal diameter without increase in external diameter. This increase in size of the lumen is so marked that the airways can be aspirated easily with a rubber catheter for removal of secretions. Exercise tests are possible with the tube in place,[42] and the catheter has been used during more than 300 thoracic surgical procedures for administration of anesthetic gases.[43-46] The rubber capillaries for inflation of the cuffs are fitted with small external control balloons designed for immediate detection of air leaks. The most conspicuous new feature of the tube is a small rubber hook placed about 4 cm. proximal to the tip of the instrument. This hook is tied flat to the tube with a piece of thread during introduction. After the tip and hook have passed by the cords, the hook is released by removal of the tie and it then engages the carina. This prevents passing the tube too far and no fluoroscopic control is required for its use. To us a most important feature is the change from a lateral opening for the right lung to an end opening with a diameter equal to that on the left. This avoids occlusion of the airway for the right lung by a deviated trachea—one of the chief difficulties encountered with the Zavod and Gebauer tubes. The largest external dimension of the Carlens tube, at the attachment of the rubber hook, is somewhat larger than that of the other two tubes. Its passage is therefore a little more difficult. The instrument was designed to be introduced by indirect laryngoscopy with a stylet (Fig. 1, Table I).

Flexible single-lumen catheters: The two internal diameters of the double-lumen catheters are limited because the single envelope, incorporating both airways, must still be small enough to be introduced without trauma to the vocal cords. Intubation of one lung only permits use of a single lumen tube of smaller external diameter than that of the double lumen catheters. At the same time a larger internal diameter provides a better airway for the intubated lung. This method, first used by Jacobaeus and associates,[1] and later advocated by Bezançon and associates,[31] was not bronchospirometry in the modern sense, since respiratory excursions of the two lungs were recorded successively rather than simultaneously. Norris and associates[47] and Bailey-Ruth[48] have converted this procedure into true bronchospirometry by the use of a face mask with two outlets. One outlet admits the tube for spirometry of the left lung. The air from the right lung passes around the tube and is collected by the face mask. Its respiratory excursions are recorded by attaching a second tube to the free aperture of the face mask.

The Norris catheter: This tube, based on the early Jacobaeus principle[1] was designed in 1948.[47] It has a single lumen, is 55 cm. long, and is made of a semi-rigid metal spiral covered by a sleeve of thin Penrose tubing (Fig. 1). It has a single inflatable cuff at the tip and is introduced with a stylet. It is advanced until the tip lies in the left main bronchus at the take-off of the left upper lobe, but not beyond. The other end of the tube emerges from the mouth through a Y-adaptor attached to the face mask. The left lung ventilates through the Norris tube. The air passage for the right lung consists of the trachea and the upper respiratory tract. The face mask collects the air from the right lung and the right spirometer is attached to the open end of the Y-adaptor. The single unquestioned advantage of this tube is that a larger airway is provided for the left lung. Therefore, resistance breathing is reduced on that side.

Comroe[49] has criticized the Norris tube on four counts. He objects to the different resistance of the airways to the two lungs, since the cross sectional area of the airway for the right lung is variable. He feels that the right lung may become obstructed if the cords close around the catheter; this was found to be the chief difficulty with the original one-lumen bronchoscope of Jacobaeus.[1] He also points out that the right lung has a larger dead space than the left lung, and that the mask may leak.

Kunkel and Lahey have devised a modification of the Norris technique which answers the latter two points of criticism.[50] Instead of a mask, an ordinary basal metabolism mouth piece is used and the Norris tube is brought out through one side of the mouth piece. A second tube of equal diameter is sealed over the remaining aperture of the mouth piece with a rubber dam and cement and serves as an airway to the right lung. A nose clamp is necessary. This modification has been used at our laboratories.

The Norris tube together with its stylet and inflatable sleeve is illustrated in Fig. 1, and its dimensions are shown in Table I.

The Bailey-Ruth bronchoscope: The use of this instrument for bronchoscopirometry was described by Braun.[48] It consists of a flexible outer shell, which is almost identical to the Norris catheter, and a narrow rigid bronchoscope which acts as core for the rubber tube. The assembled instrument, which is provided with a light at the tip, is introduced like a bronchoscope. When the tip has been correctly placed under direct vision, the rigid bronchoscope is removed, leaving the soft single-lumen catheter in place. Air from the other lung is apparently collected with a mask. We have had no opportunity to see this device.

Bronchospirometry in the experimental animal: Although other techniques, such as plugging of individual bronchi with malleable plastics,[51] and surgical production of a "small lung" by exteriorization of a lobar bronchus,[52] are still occasionally used for special studies, the principle of bronchospirometry has been almost universally accepted for animal work. The smaller diameter of the animals' airway presents a special problem since tubes must be made of materials of a thickness equal to that used in man. The greater mobility of the mediastinum, especially in dogs, and the higher take-off of the upper lobe bronchi present additional problems. Because of these difficulties, Wright,[53] Boutourline-Young[54] and others have preferred double bronchoscopes made of thin metal. Unlike the Jacobaeus bronchoscope, the two airways are of equal length and the tip is fitted with specially constructed double rubber cuffs. The cuffs are inflated and airtight contact is made by applying pressure against the carina. Liebow and associates[55] have modified the Van Allen cannula[56] for bronchospirometry. The flexible double-lumen tubes of Gebauer and of Carlens, designed for use in man, have also been successfully employed in dogs.[30, 57, 58]

Peroral intubation of this type permits preservation of the animal for study at a future date. This was a basic requirement in many of the experiments described.[55, 57, 58] If the animal can be sacrified, tracheotomy or complete transection of the trachea simplifies the procedure. This is almost a necessity if animals smaller than dogs are to be used. A variety of techniques of this type were developed before 1932 and have been reviewed by Björkman.[2] More recent methods were collected by Heemstra in 1948.[59] At present the trachea divider of Dirken and van Dishoeck appears to be popular for small animals.[60]

Investigations of Cardiorespiratory Physiology.—Normal division of pulmonary function: The number of normal volunteers who have been studied by bronchospirometry is small. In ten observations, Björkman found an average function of 54 to 55 per cent of the total on the right side.[2] Most investigators have agreed with these results, no matter what bronchoscope or tube was used.[37, 61, 62] Steinmann suspected that the lesser function of the left lung is an artefact due to the greater length of the narrow airway on the left.[63] Baldwin and associates gave 50 to 65 per cent as limits of normal functional participation for the right lung.[64] We have considered quantitatively the problem brought up by Steinmann and have come to the conclusion that the normal limits of function given by Baldwin are correct.[3] The functional contribution of the two lungs varies considerably from individual to individual although it does not matter, in any given normal individual, whether that contribution is measured by oxygen uptake, ventilation or vital capacity.

Bronchospirometry and the pulmonary blood supply: Maar, using turtles and rabbits, demonstrated nearly fifty years ago that partial occlusion of one branch of the pulmonary artery reduced that lung's oxygen uptake and carbon dioxide output.[65] The decrease in gaseous exchange was roughly proportional to the degree of occlusion and was accompanied by a corresponding increase of exchange of the uninvolved lung. Later, Churchill and Agassiz

extended these experiments to observations of the effect of pulmonary arterial and venous occlusion on ventilation.[9] A pericardial window operation permitted manipulation of the hilar structures during bronchospirometry without complicating pneumothorax. Liebow and associates, in a series of brilliant investigations, explored the effect of chronic unilateral pulmonary arterial occlusion on the collateral circulation of the lung.[55] The fraction of the total left cardiac output diverted for perfusion of the involved lung could be calculated. This bronchial arterial supply increased slowly over the course of months and even years.

The effect of acute unilateral pulmonary arterial occlusion in the intact animal was recently described by Dotter and Lukas[29] and by Carlens and associates.[30] Arterial flow to one lung was interrupted with a cardiac catheter fitted with an inflatable balloon. Right ventricular or common pulmonary arterial pressures were recorded with a separate catheter. Dotter and Lukas noted a marked increase in right ventricular pressure; the procedure was poorly tolerated and was accompanied by electrocardiographic changes. This was probably because the common pulmonary artery had been partially or completely occluded in a number of their dogs. Of greater interest were the studies of Carlens, because a number of occlusions were performed during bronchospirometry and the procedure was performed in man as well as in dogs. A minimal increase of pulmonary arterial pressure in their experiments was soon compensated for, and the procedure was well tolerated. Bronchospirograms showed a cessation of gaseous exchange on the side of occlusion and a resumption of normal oxygen uptake when the balloon was deflated. These striking tracings were similar in appearance to those obtained in a patient whose left pulmonary artery had been surgically ligated[66] and of another who was born without a right branch of the pulmonary artery.[67]

The effect of various gas mixtures during bronchospirometry: Most studies, involving the supply of different gases to the two lungs, were in one way or another related to investigations of pulmonary blood supply. The calculation of effective bronchial blood flow in dogs[55] and in man[67] required breathing of gases with different oxygen tensions by the two lungs. Björkman applied the acetylene method for the determination of cardiac output to bronchospirometry. He was able to show that (1) in individuals with normal or nearly normal lungs the acetylene method is applicable; (2) the cardiac output to the right and left lungs is roughly proportional to their relative oxygen uptake; and (3) in the presence of pulmonary pathology this method for determination of cardiac output is not reliable since it depends on nearly instantaneous mixing of inspired gases.[68] He was the first to point out that, in the absence of hypoxia, the differential cardiac output must be the same as the differential oxygen uptake. This concept was later used by Dirken and Heemstra in a study of the effect of alveolar oxygen tension on pulmonary blood flow.[69, 70] By supplying one lung of rabbits with an oxygen-poor atmosphere they showed that the blood flow decreases in areas of the lung where alveolar oxygen tension is low. These results were confirmed by Rahn and Bahnson who demonstrated in dogs that nitrogen breathing of one lung causes a reduction of blood flow on that side within a few minutes.[71] The occurrence of rapid adjustments of this nature was given support by results of ambient air and oxygen bronchospirometry in man performed in the presence of pulmonary disease.[5, 42]

A number of other studies, involving the breathing of different gases by the two lungs, were carried out in man. Jacobaeus and Bruce used oxygen for one lung and room air, nitrogen, or nitrogen with 5 per cent carbon dioxide for the other. They studied the control of respiration, the excretion of carbon dioxide, and differential distribution of blood to the lesser circulation.[13, 14] Whitehead and associates supplied air to one lung and a low oxygen mixture to the other during an investigation of the adaptive mechanism of normal and diseased lungs.[72, 73] Wright and Woodruff subjected one lung to nitrogen breathing to simulate conditions occurring during collapse therapy.[74] The increase in ventilation of the opposite lung was not closely related to the severity of disease of the lung breathing nitrogen, and was unrelated to the amount of oxygen uptake lost due to contralateral nitrogen breathing.

A number of investigators have suggested that the use of almost pure oxygen during closed spirometry may obscure the insufficiency of the diseased lung.[32, 42, 72-74] To avoid

this, Whitehead[73] and Wright[74] have used an open circuit technique permitting a constant supply of room air to the lungs. Arnaud and associates[32] interposed boxes of 200-liter capacity in the inspiratory circuit of an ordinary spirometer to slow changes in gas composition occurring during closed spirometry. This permitted essentially room air breathing for short periods of time. This technique was applied to bronchospirometry by Tulou.[34] We have shown that a severely damaged lung often participates to a greater extent in the total oxygen uptake when breathing pure oxygen instead of room air.[75, 76] A device which makes possible the simultaneous graphic recording of respiratory excursions while breathing room air will be described in a later paper of this series.[5]

Unilateral bronchial occlusion and atelectasis: The effect of occlusion of the airway to one lung has been explored. The work of Arnaud,[32] Tulou[34] and Rogers[36] has been referred to. Jacobaeus and Bruce found that unilateral occlusion is usually well tolerated. The response varied slightly with the phase of the respiratory cycle where the air stream was interrupted.[13] An increase in cardiac output occurred immediately after occlusion. They felt that partial atelectasis would reduce the stress of occlusion since it should be accompanied by a shunting of the blood to the opposite side. Björk and Salen investigated the mechanism of this shunting in dogs.[58] To produce atelectasis it was necessary to supply pure oxygen to that lung for some time before occlusion and forceful evacuation of air was necessary prior to obliteration of the airway. Angiocardiography revealed that, even in the presence of total atelectasis, many hours and even days were required before all of the blood flow had been shunted to the other lung. Bence and associates produced unilateral atelectasis in man by forceful withdrawal of 100 ml. of air from one lung with a bronchospirometric catheter after fullest expiration.[77] Striking roentgenograms with all the characteristics of atelectasis were obtained. The collapse was immediately reversible and apparently no ill effects were suffered by the patients who volunteered for this study.

The mobility of the mediastinum in man: It is generally thought that the human mediastinum is much more rigid than that of most experimental animals. It is known that this rigidity is increased by parenchymal and particularly by pleural disease. A number of bronchospirometric studies have shown that the mobility of the human mediastinum is often underestimated. Bronchospirometry during administration of artificial pneumothorax, in patients without pleural infection, showed that only one-fifth of the injected air is exhaled by the lung on that side.[78, 79] Some of the injected air increased the pneumothorax space by permitting the rib cage to distend and by depressing the diaphragm on that side. However, most of the increase of the space was due to shift of the mediastinum to the opposite side. The diminution of the volume of the contralateral lung due to this mediastinal shift was not recorded quantitatively. Separate determination of the residual volume and vital capacity of each lung before and after induction of therapeutic pneumothorax in one patient revealed a reduction of 510 ml. in the total lung capacity of the involved side and a reduction of 310 ml. on the uninvolved side.[6] Sarnoff, Gaensler, and Maloney applied external electrical stimulation to one phrenic nerve during bronchospirometry.[80] They found that, in the absence of all other respiratory efforts, the human mediastinum may swing sufficiently with each breath to cause normal ventilation of the lung on the nonstimulated side.

The effect of changes in position on differential function: Apparently, few who have used the bronchospirometric technique for any extent of time have been able to resist the temptation to study the effect of the lateral decubitus position on differential ventilation and oxygen uptake. Results have shown consistently that a lung absorbs a greater percentage of the total oxygen uptake when it is in the lower position of the lateral decubitus.[2, 24, 25, 63, 81, 82] We agree with Van Meeteren that many explanations which have been offered for the increase in oxygen uptake of the lower lung are unsatisfactory.[83] We feel that the answer lies in a shunting of blood from the upper lung to the lower, as pulmonary vascular resistance is altered by the change in the degree of inflation of the two lungs.

A relative increase of ventilation by the lung in the dependent position was reported occasionally. It was a surprising finding, in view of Vaccarezza's roentgenologic demonstration

that the lower lung occupies a smaller volume in all positions of the respiratory cycle and shows smaller respiratory excursions.[24, 25] Björk feels that increased ventilation is due to the fact that the inferior diaphragm moves up into the thorax in an extreme expiratory position. This, he thinks, should facilitate larger inspiratory movements which more than compensate for the decreased rib expansion of the lower hemithorax.[84] Steinmann found the increase of oxygen uptake, of ventilation and even of vital capacity of the lower lung such a constant finding that he used the lateral decubitus position during his routine bronchospirometries as a functional test.[63] He concluded that, if an impaired lung, upon being placed in the dependent position of the lateral decubitus, did not increase its relative share of function, its reaction to stress and its reserve is very poor.

Observations on the effect of other mechanical factors on differential function might well be mentioned here. Compression of one hemithorax by sandbags did not alter differential function[81] and filling of the stomach with air[2] or raising one arm[2] also caused no appreciable shift of function. Injection of 1,000 ml. of water into the stomach of a patient with a hiatus hernia brought about a severe diminution of the functional participation of the left lung.[7] Rothstein and Strzelczyk examined eight patients who had learned "unilateral breathing"; they found that this voluntary exercise was not reflected by an increase in functional participation of the lung on that side.[85] Jacobaeus[15] and Björkman[2] were much impressed by the ineffectiveness of the coughing effort of diseased lungs. Their findings were striking although only partly applicable because resistance breathing and spirometer inertia blunted the peaks of the rapid expiratory efforts and the coordinated action of the expiratory muscles and vocal cords was impaired when the cords were rendered immobile by the double bronchoscope.

Differential alveolar air collection, differential cardiopneumography and differential pneumotachography: Vaccarezza and associates collected alveolar air from the two lungs separately.[23, 86, 87] Results of these studies were not fruitful from an investigative standpoint and added little to the clinical evaluation of unilateral pulmonary disease. Janton and associates obtained some evidence by this technique that no gaseous exchange took place on the left side in a patient whose left pulmonary artery had been accidentally ligated.[88] Gaensler and Cugell applied the open circuit oxygen dilution method for determination of residual volume during bronchospirometry.[6] The nitrogen content of the alveolar air was analyzed after oxygen breathing: the "alveolar mixing index" or residual nitrogen content was often much larger on the diseased side. Rahn and Bahnson were able to perform continuous and simultaneous alveolar air analysis of each lung during bronchospirometry in the dog.[71] Their technique was not described.

Differential cardiopneumatic movements have been recorded; it was felt that this permitted more detailed analysis of oscillations due to pressure changes in the heart and great vessels.[23, 89]

The recording of air flow curves of the two lungs by differential pneumotachography would be of great interest because a diagnosis of unilateral obstruction or emphysema might be made. A reference to this technique was made in an illustration shown by Proctor and Hardy but there was no mention of this in their text.[90] We have used a twin pneumotachograph apparatus designed according to Silverman and Whittenberger.[91] Resistance breathing with the older bronchospirometric catheters caused such abnormal pneumotachograms that all but the most extreme pulmonary changes were masked by the effect of the tube.[3] More recent experiences with this instrument used in conjunction with the Carlens catheter will be reported subsequently.

Other experimental studies: Many investigators have pointed out that metabolism during bronchospirometry can never be basal. Cardiac output is increased, on the average, by about 49 per cent.[68] Exercise tests during bronchospirometry have generally not been successful because of the marked narrowing of the airway imposed by the catheter. With improved tubes it was recently possible to study differential function during exercise in the supine position on a bicycle ergometer.[12] This effort, in twenty-two patients, caused a 150 to 550 per cent increase of oxygen uptake. Suprisingly, the ratio of oxygen uptake be-

tween the two lungs was the same during exercise and at rest even when the damaged lung contributed as little as 3 per cent of the total oxygen uptake. It was concluded that, during exercise, the blood supply increases in the same proportion in the functioning parenchymal tissue of the diseased lung as in the well lung.

Lanari and associates have transplanted freshly resected lungs from some dogs into others who had just had pneumonectomy.[57] Bronchospirometry, two weeks later, showed that the transplanted lungs were responsible for as much as 25 to 37 per cent of the total oxygen uptake.

Clinical Bronchospirometry.—Abnormal pulmonary arterial supply: Unilateral deficiency of pulmonary arterial blood flow apparently affects only oxygen uptake. This was first observed by Jacobaeus in a patient who showed partial obstruction of the right branch of the pulmonary artery by lymph nodes.[16] Accidental surgical occlusion of the left branch of the pulmonary artery was followed by poor gaseous exchange on the same side in one case,[88] and appeared to affect oxygen uptake without disturbing ventilation and vital capacity in another case.[66] Madoff, Strieder, and Gaensler have studied a patient with congenital absence of the right branch of the pulmonary artery, discovered by angiocardiography.[67] The right lung was responsible for over 50 per cent of the total ventilation and vital capacity, but took up no oxygen while breathing room air and only a very slight amount while breathing pure oxygen.

Diffuse pulmonary disease: Bronchospirometric studies of diffuse pulmonary disease, particularly bronchial asthma and emphysema, have generally not been fruitful.[17, 92, 93] Although the procedure is usually difficult in asthmatics, we were able to obtain several satisfactory tracings even when an attack was induced during bronchospirometry by intravenous administration of histamine or methacholin.[93, 94] Suggestive evidence was obtained that bronchospasm and/or bronchial mucosal edema due to asthma may occasionally occur predominantly on one side.[93] Wright and Filley reported bronchospirometric studies in patients with pulmonary fibrosis.[95] However, only those patients were selected for the procedure where the disease was localized rather than diffuse.

Predominantly unilateral pulmonary disease: It is difficult to organize the results of the many studies where a few examples of the effect of a particular disease on differential function were shown. The greatest unanimity of opinion exists concerning the ill effects of pleural involvement on pulmonary function.[15, 81, 96-100] Pinner and associates stated that in their series of over 200 cases all lungs showing less than 25 per cent of the total oxygen uptake had some pleural disease, while purely parenchymal disease usually caused only an insignificant decrease of function.[81] We agree with this but would add that unilateral endobronchial disease may have equally damaging effects on function.[94, 101] The only universal finding which applies to unilateral parenchymal disease is that oxygen uptake suffers more than other measurable function. Ventilation is often normal in lungs with little involvement and is never as greatly reduced as oxygen uptake, no matter how severe the disease.[2, 15, 61] On the contrary, we have frequently observed that lungs with moderate or advanced disease and severe reduction of oxygen uptake ventilate *more than their normal share at rest.*[100]

The bronchospirometric technique does not appear to be sensitive enough to show a functional loss due to early cavitation.[15] Large cavities cause considerable loss of oxygen uptake while ventilation and carbon dioxide output often remain unimpaired.[15, 63] The measurable functional impairment due to air cysts and bullae depends on their size and on the patency of the bronchial communication.[16, 63, 102] In addition to pulmonary tuberculosis, in all its forms and with all its complications, a number of other diseases have been studied, though in smaller numbers. They include bronchiectasis,[7, 15, 63, 103] lung abscess,[2, 15, 25, 53] lung cysts,[16, 92, 102] actinomycosis,[63] sarcoidosis,[7] pleural effusion and empyema,[96-100] and primary and metastatic carcinoma.[7, 15, 63, 75] A brief investigation of the differential functional effect of kyphoscoliosis and its correction by corsets has been reported.[63]

Medical and surgical collapse and resection: The greatest clinical importance of bronchospirometry is in the study of bilateral pulmonary disease and the effect of collapse therapy and thoracic surgical procedures. Pinner has pointed out that bronchospirometry is

the only method which permits study of the effect of pulmonary collapse on the involved lung, as well as observation of the compensatory mechanism of the contralateral lung.[104] The effect of therapeutic pneumothorax has been studied most intensively because it was the popular collapse procedure when bronchospirometry became more widely used.[2, 7, 15, 34, 48, 78, 104-108] The marked loss of function characteristic of active pneumothorax therapy was partially responsible for the gradual decline in popularity of this procedure. There have been few opportunities to study the return of function after uncomplicated re-expansion of successful pneumothorax. Patients at that time are seldom hospitalized, and over the years, the interest of investigators has usually shifted to other fields. A very poor function of the re-expanded pneumothorax lung has often been described. Some bronchospirometric data suggest that obliteration of the pneumothorax space occurs chiefly by mediastinal and diaphragmatic shift and partial collapse of the hemithoracic cage rather than by true re-expansion of the lung.[61] Others have felt that the degree of re-expansion is so variable that any prediction of the functional end result is impossible.[53]

In sharp contrast to intrapleural pneumothorax, the loss of function caused by extrapleural pneumothorax was shown to be very small by Vaccarezza and associates[109] and by Gaensler and Strieder.[110] Similar small losses of function were found after extrapleural plombage by Dressler and associates.[111] We have compared the loss of function due to extrapleural pneumothorax with that due to other collapse and resection therapy and have concluded that only phrenic interruption occasionally rivals extrapleural pneumothorax in its function sparing characteristics.[110]

Some disagreement exists concerning the effect of phrenic interruption but it is generally thought that the functional loss is small.[53, 80, 110, 112] However, the loss may be considerable, if diaphragmatic fixation occurs high in the chest.

A number of bronchospirometric studies have been recorded before and after thoracoplasty.[2, 15, 61, 110, 113, 114] In general, the variations in extent of disease, number of ribs removed, number of stages, and contralateral disease or collapse have been so great that no definite conclusions were possible. We agree with Jacobaeus that function on the side of thoracoplasty may slowly improve over a period of as long as three years following the initial loss due to the operation. Both Pinner and associates[61] and Björkman[2] have shown that thoracoplasty diminishes the function of both lungs to some extent.

Some of the effects of lobectomy have been studied, but the number of cases was usually too small to warrant any comparison of surgical collapse with lobar resection.[103, 110] Kay and associates studied twelve patients after lower lobe lobectomy and concluded that temporary phrenic interruption favorably influences the functional future of the remaining lobe.[115] Bronchospirometric examination prior to pneumonectomy is useful for estimation of probable loss of function due to resection.[75, 110, 116, 117] Differential residual volume determination has made it possible to observe the total lung capacity and resting volume of the remaining lung even before pneumonectomy. Conclusions can then be drawn concerning the effect of pneumonectomy on the remaining lung.[75]

Pulmonary decortication, though primarily designed to obliterate infected space, stands apart from other surgical procedures in that it generally increases rather than decreases function. Results have been variable but it appears that lungs with little parenchymal involvement prior to collapse generally show a good return of function. This has been true after decortication for traumatic hemothorax and for unexpandable pneumothorax lungs. Massive parenchymal involvement preceding tuberculous or mixed empyema usually has prevented good return of function.[96-100] Poudrage for the treatment of recurrent spontaneous pneumothorax has little effect on function, at least within the first year after operation.[118]

Two other publications on bronchospirometry are related to surgery but not to function. Björk, Carlens and Crafoord have described the use of a bronchospirometric tube for endobronchial anesthesia during pulmonary resection for suppurative disease,[43] and for open bronchus closure and resection of the carina and tracheal wall.[44] They found that spreads could be avoided by supplying anesthetic gas to the good lung and by applying constant or

intermittent suction to the diseased lung. Cincotti and Watson[45] and the author have applied this technique successfully. Differential functional analysis during thoracic surgical procedures will be described in a subsequent paper of this series.[8]

Technique of bronchospirometry: Some papers which deal, in part, with the technique of bronchospirometry should be mentioned. Most of them have already been cited in another connection.[1, 2, 25, 38-41, 47, 64, 81, 119-121] Björkman's is the most exhaustive and Leiner and Liebler's and Wright and Michelson's are more recent and very helpful. References concerning indications, contraindications and complications of bronchospirometry will be cited in connection with our own experiences.[4]

SUMMARY

The literature on bronchospirometry was reviewed from 1934 to 1951.

A number of bronchospirometric catheters and tubes were described and their dimensions and special characteristics were compared.

REFERENCES

1. Jacobaeus, H. C., Frenckner, P., and Björkman, S.: Some Attempts at Determining the Volume and Function of Each Lung Separately, Acta Med. Scandinav. **79**: 174, 1932.
2. Björkman, S.: Bronchospirometrie, eine klinische Methode die Funktion der menschlichen Lungen getrennt und gleichzeitig zu untersuchen, Acta Med. Scandinav. Suppl. 56, 1934.
3. Gaensler, E. A., Maloney, J. V., Jr., and Björk, V. O.: Bronchospirometry. II. Experimental Observations and Theoretical Considerations of Resistance Breathing, J. Lab. & Clin. Med. **39**: 935, 1952 (this issue).
4. Gaensler, E. A., and Watson, T. R., Jr.: Bronchospirometry. III. Complications, Contraindications, Technique and Interpretation, J. Lab. & Clin. Med. to be published.
5. Gaensler, E. A., and Cugell, D. W.: Bronchospirometry. IV. Ambient Air and Oxygen Recording Bronchospirometry. In preparation.
6. Gaensler, E. A., and Cugell, D. W.: Bronchospirometry. V. Differential Residual Volume Determination. In preparation.
7. Gaensler, E. A., and Watson, T. R., Jr.: Bronchospirometry. VI. Analysis of Results of 1,000 Examinations. In preparation.
8. Cincotti, J. J., Watson, T. R., Jr., and Gaensler, E. A.: Bronchospirometry. VII. Differential Function During Thoracic Surgical Procedures. In preparation.
9. Churchill, E. D., and Agassiz, A.: A Method for Separating the Air Breathed by the Right and Left Lungs, Together With the Effect of Pulmonary Circulatory Changes on This Divided Breathing, Am. J. Physiol. **76**: 6, 1926.
10. Löwy, A., and Schrötter, v. H.: Untersuchungen über die Blutzirculation beim Menschen, Ztschr. f. exper. Path. u. Therap. **1**: 197, 1905.
11. Hess, R.: Über die Durchblutung nicht atmender Lungengebiete, Deutsch. Arch. f. klin. Med. **106**: 478, 1912.
12. Frenckner, P.: Bronchial and Tracheal Catheterization and Its Clinical Applicability, Acta oto-laryng. Suppl. 20, 1934.
13. Jacobaeus, H. C., and Bruce, T.: A Bronchospirometric Study on the Ability of the Human Lungs to Substitute for One Another, I., Acta med. Scandinav. **105**: 193, 1940.
14. Jacobaeus, H. C., and Bruce, T.: A Bronchospirometric Study on the Ability of the Human Lungs to Substitute for One Another, II., Acta med. Scandinav. **105**: 211, 1940.
15. Jacobaeus, H. C.: Bronchospirometry, J. Thoracic Surg. **7**: 235, 1938.
16. Jacobaeus, H. C.: Ergebnisse der Bronchospirometrie, Schweiz. med. Wchnschr. **66**: 865, 1936.
17. Brighton, G. R., and Barach, A. L.: Bronchospirometry, Ann. Otol., Rhin. & Laryng. **47**: 1061, 1938.
18. Warring, F. C., Jr.: Ventilatory Function. Experiences With a Simple Practical Procedure for Its Evaluation in Patients With Pulmonary Tuberculosis, Am. Rev. Tuberc. **51**: 432, 1945.
19. Cohen, A. G., and Geffen, A.: Roentgenographic Methods in Pulmonary Disease, Am. J. Med. **10**: 375, 1951.
20. Vaccarezza, R. F., Lanari, A., and Soubrie, A.: Discrepancies Between Clinical-radiological and Bronchospirometric Findings, Am. Rev. Tuberc. **55**: 128, 1947.

21. Johns, E. L., and Seabury, J. H.: The Correlation Between Clinical Estimation and Laboratory Determination of Functional Pulmonary Capacity, New Orleans M. & S. J. **102**: 10, 1949/50.
22. Birath, G., and Källqvist, I.: Double Exposure Grid for Registering Respiratory Movements in Two Phases on the Same Film, Nord. med. **43**: 427, 1950.
23. Birath, G., and Crafoord, C.: Function Tests in Pulmonary Surgery, J. Thoracic Surg. **22**: 414, 1951.
24. Vaccarezza, R. F., Lanari, A., Bence, A. E., and Labourt, F.: Influencia del decubito lateral sobre el reposo pulmonar: Estudio funcional de cada pulmon por separado, An. Cated. de pat. y clin. tuberc. **3**: 254, 1941.
25. Vaccarezza, R. F., Bence, A., Lanari, A., Labourt, F., Segura, R. G.: The Study of the Two Lungs Separately in Practical and Research Work, Dis. Chest **9**: 95, 1943.
26. Simonin, P., Girard, J., Sadoul, P., Grilliat, J. P., and Braun, P.: L'exploration séparées des poumons par bronchospirométrie; étude critique d'aprée 80 séparations, Rev. Med. de Nancy **75**: 250, 1950.
27. Labesse, P.: Une nouvelle méthode d'exploration fontionnelle des poumons séparés, Rev. de la Tuberc. **10**: 153, 1946.
28. Anthony and Hansen: Die Bewegung der Thoraxwand bei einseitigen Lungen- und Pleuraerkrankungen, Beitr. z. Klin. Turberk. **72**: 221, 1929.
29. Dotter, C. T., and Lukas, D. A.: Acute Cor Pulmonale. An Experimental Study Utilizing a Special Cardiac Catheter, Am. J. Physiol. **164**: 254, 1951.
30. Carlens, E., Hanson, H. E., and Nordenstrom, B.: Temporary Unilateral Occlusion of the Pulmonary Artery. A New Method of Determining Separate Lung Function and of Radiologic Examination, J. Thoracic Surg. **22**: 527, 1951.
31. Bezançon, F., Braun, P., Soulas, G., Cachin, M., and Guillaumin, N.: La division des airs, examen functionnel des poumons séparés, Bull. Acad. de med., Paris **115**: 12, 1936.
32. Arnaud, J., Tulou, P., and Merigot, R.: L'exploration de la fonction respiratoire, Paris: Masson et Cie, 1947, pp. 208-228.
33. Fury, L.: Successive Bronchospirometry in Active Pulmonary Therapy, Rozhledy v. tuberk. **9**: 62, 1949.
34. Tulou, P.: La Broncho-spirométrie avec sonde souple. Methode d'exploration fonctionelle des poumons séparés, Rev. de la tuberc. **11**: 543, 1947.
35. Morera, J.: La Bronchospirometria en las afecciones de las vias respiratorias, Arch. Soc. Estud. Clin. Habana **42**: 283, 1949.
36. Rogers, W. L.: in discussion of Leiner, G., Pinner, M., and Zavod, W. A.: Bronchospirography. II. Application to Collapse Therapy, J. Thoracic Surg. **10**: 42, 1940.
37. Breton, A., and Méréau, J.: Intérêt des épreuves fonctionnelles respiratoires par airs séparés en chirurgie thoracique, Le Poumon **3**: 335, 1947.
38. Gebauer, P. W.: A Catheter for Bronchospirometry, J. Thoracic Surg. **8**: 674, 1939.
39. Zavod, W. A.: Bronchospirography. I. Description of the Catheter and the Technique of Intubation, J. Thoracic Surg. **10**: 27, 1940-41.
40. Carlens, E.: A New Flexible Double-lumen Catheter for Bronchospirometry, J. Thoracic Surg. **18**: 742, 1949.
41. Wright, G. W., and Michelson, E.: Bronchospirometry, in Methods in Medical Research, Vol. II, Comroe, J. H., Jr., ed., Chicago, 1950, The Year Book Publishers, Inc., pp. 82-93.
42. Björkman, S., and Carlens, E.: The Lung Function During Rest and Exercise in Lung Diseases: A Bronchospirometric Study, Acta Med. Scandinav. Supp. **259**: 63, 1951.
43. Björk, V. O., and Carlens, E.: The Prevention of Spread During Pulmonary Resection by the Use of a Double-lumen Catheter, J. Thoracic Surg. **20**: 151, 1950.
44. Björk, V. O., Carlens, E., and Crafoord, C.: The Open Closure of the Bronchus and the Resection of the Carina and of the Tracheal Wall, J. Thoracic Surg. **23**: 419, 1952.
45. Cincotti, J. J., and Watson, T. K., Jr.: Personal communications.
46. Harken, D. E.: Personal communications.
47. Norris, C. M., Long, J., and Oppenheimer, M. J.: Bronchospirography: Apparatus and Technique, J. Thoracic Surg. **17**: 357, 1948.
48. Braun, P.: L'exploration fonctionnelle des poumons séparés par bronchospriométrie, Nancy, Société d'Impressions Typographiques, 1951.
49. Comroe, J. H., Jr.: Commenting on Wright, G. W., and Michelson, E.: Bronchospirometry, in Methods of Medical Research, Vol. II, Comroe, J. H., Jr., ed., Chicago, 1950, The Year Book Publishers, Inc., p. 92.
50. Kunkel, P., and Lahey, W. J.: Personal communications.

51. Bross, W., and Luecken, B.: Les troubles de la circulation du sang et du volume respiratoire au cours des opérations sur les poumons et le problème du mécanisme nerveux du choc operatoire, Rev. de chir., Paris **76**: 648, 1938.
52. Jazbasic, D. M.: "Kleine Lunge," eine neue Möglichkeit für die Erforschung der Lungenfunktion, Beitr. z. Klin. Tuberk. **96**: 265, 1941.
53. Wright, G. W.: Personal communications.
54. Boutourline-Young, H. J.: Personal communications.
55. Liebow, A. A., Hales, M. R., Harrison, W., Bloomer, W., and Lindskog, G. W.: The Genesis and Functional Implications of Collateral Circulation of the Lungs, Yale J. Biol. & Med. **22**: 637, 1950.
56. Van Allen, C. M.: A Dilatable Bronchial Cannula, Yale J. Biol. & Med. **2**: 295, 1930.
57. Lanari, A., Molins, M., and Croxatto, O.: Homoinjertos de Pulmon en Perros. Tecnica y resultados funcionales y anatomicos, Medicina, Buenos Aires **11**: 12, 1951.
58. Björk, V. O., and Salen, E. F.: The Blood Flow Through an Atelectatic Lung, J. Thoracic Surg. **20**: 933, 1950.
59. Heemstra, H.: Med. Dissertation, Groningen, 1948.
60. Dirken, M. N. J., and van Dishoeck, H. A. E.: Gibt es eine periphere Atemregulation durch Kohlensäure? Arch. f. d. ges. Physiol. **238**: 713, 1937.
61. Pinner, M., Leiner, G., and Zavod, W. A.: Bronchospirometry. III. The Functional Capacity of Normal Lungs, Severely Damaged Lungs, Lungs With Strictly Parenchymal Lesions, Thoracoplasty Lungs and Re-expanded Pneumothorax Lungs, J. Thoracic Surg. **11**: 241-65, 1942.
62. Vaccarezza, R. F., Lanari, A., Bence, A. E., and Labourt, F.: Examen funcional de cada pulmon por separado en tisiologia, An. Cated. de pat. clin. y tuberc. **3**: 5, 1941.
63. Steinmann, E. P.: Zur Frage der Bronchospirometrie, Praxis **38**: 799, 1949.
64. Baldwin, E. de F., Cournand, A., and Richards, D. W., Jr.: Pulmonary Insufficiency. I. Physiological Classification, Clinical Methods of Analysis, Standard Values in Normal Subjects, Medicine **27**: 243, 1948.
65. Maar, V.: Weitere Untersuchungen über den Einfluss der die Lungen passierende Blutmenge auf den respiratorischen Stoffwechsel derselben, Scandinav. Arch. f. Physiol. **16**: 358, 1904.
66. Roh, C. E., Greene, D. G., Himmelstein, A., Humphreys, G. H., and Baldwin, E. de F.: Cardiopulmonary Function Studies in a Patient With Ligation of the Left Pulmonary Artery, Am. J. Med. **6**: 795, 1949.
67. Madoff, I. M., Strieder, J. W., and Gaensler, E. A.: Congenital Absence of the Right Pulmonary Artery Diagnosed by Angiocardiography and Cardiorespiratory Studies, New England M. J., 1952.
68. Björkman, S.: Anwendbarkeit der Acetylenmethode bei pathologischen Zuständen in den Lungen, Beitr. z. Klin. Tuberk. **88**: 519, 1936.
69. Dirken, M. N. J., and Heemstra, H.: Alveolar Oxygen Tension and Lung Circulation, Quart. J. Exper. Physiol. **34**: 193, 1947/8.
70. Dirken, M. N. J., and Heemstra, H.: The Adaptation of the Lung Circulation to the Ventilation, Quart. J. Exper. Physiol. **34**: 213, 1947/8.
71. Rahn, H., and Bahnson, H. T.: Simultaneous Determination of Blood Flow Through Each Lung, Fed. Proc. **9**: 102, 1950.
72. Whitehead, W. K., O'Brien, E. J., and Tuttle, W. M.: Studies of Individual Lung Function. I. An Open-circuit Procedure With Air and Low-oxygen Mixtures as the Inspired Gas, J. Thoracic Surg. **11**: 266, 1942.
73. Whitehead, W. K., Whitman, N., and Shacter, B.: The Functional Capacity of the Contralateral Lung, Am. Rev. Tuberc. **47**: 173, 1943.
74. Wright, G. W., and Woodruff, W.: Bronchospirography. Ventilation and Oxygen Absorption of Normal and Diseased Lungs During Nitrogen Respiration in the Opposite Lung, J. Thoracic Surg. **11**: 278, 1941.
75. Gaensler, E. A., and Strieder, J. W.: Progressive Changes in Pulmonary Function After Pneumonectomy. The Influence of Thoracoplasty, Pneumothorax, Oleothorax and Plastic Sponge Plombage on the Pneumonectomy Side, J. Thoracic Surg. **22**: 1, 1951.
76. Gaensler, E. A., and Cugell, D. W.: Ambient Air Recording Bronchospirometry and Differential Residual Volume Determination, J. Clin. Investigation **30**: 639, 1951.
77. Bence, A., and Lanari, A.: Atelectasia pulmonar por aspiracion del aire residual, Medicina, Buenos Aires **2**: 152, 1942.
78. Björkman, S.: Bronchospirometriska studier vid pneumothorax, Nord. Med. Tidskrift **10**: 1964, 1935.
79. Bence, A. E., Lanari, A., and Rodriguez, E. J.: Relacion entre el volumen del aire insuflado en la camara pleural y la disminucion del volumen pulmonar, Medicina, Buenos Aires **8**: 16, 1948.

80. Sarnoff, S. J., Gaensler, E. A., and Maloney, J. V.: Electrophrenic Respiration. IV. Differential Bronchospirometry During Stimulation of One Phrenic Nerve in Man, J. Thoracic Surg. **19**: 929, 1950.
81. Pinner, M., Leiner, G. C., and Zavod, W. A.: Bronchospirometry, Ann. Int. Med. **22**: 704, 1945.
82. Rothstein, E., Landis, F. B., and Narodick, B. G.: Bronchospirometry in the Lateral Decubitus Position, J. Thoracic Surg. **19**: 821, 1950.
83. Van Meeteren, A.: Bronchospirometrie, Nederl. tijdschr. v. geneesk. **80**: 4795, 1936.
84. Björk, V. O.: Personal communications.
85. Rothstein, E., and Strzelczyk, R.: Voluntary "Unilateral Breathing," Ann. Int. Med. **34**: 401, 1951.
86. Bence, A. E., Labourt, F., and Lanari, A.: Concentracion del anhidrido carbonico en el aire residual de sujetos normales, An. cated. de pat. y clin. tuberc. **3**: 361, 1941.
87. Vaccarezza, R. F., Lanari, A., Soubrie, A., and Labourt, F.: Composicion del aire alveolar de cada pulmon en individuos normales y en tuberculosos pulmonares, Medicina, Buenos Aires **3**: 145, 1943.
88. Janton, O. H., Redondo, H. P., and Scott, J. C.: Pulmonary Gas Exchange Following Ligation of a Pulmonary Artery in Man, Federation Proc. **7**: 61, 1948.
89. Bence, A. E., Segura, R. G., and Lanari, A.: Los movimientos cardioneumaticos de cada pulmon, An. Cated. de pat. y clin. tuberc. **7**: 5, 1945.
90. Proctor, D. F., and Hardy, J. B.: Studies of Respiratory Air Flow. I. Significance of the Normal Pneumotachogram, Bull. Johns Hopkins Hosp. **85**: 253, 1949.
91. Silverman, L., and Whittenberger, J. L.: Clinical Pneumotachograph, in Methods in Medical Research, Vol. II, Comroe, J. H., Jr., ed., Chicago, 1950, The Year Book Publishers, Inc., pp. 104-112.
92. Gaensler, E. A., and Carter, M. G.: Ventilation Measurements in Pulmonary Emphysema Treated With Pneumoperitoneum, J. Lab. & Clin. Med. **35**: 945, 1950.
93. Gaensler, E. A., commenting on, Blades, B., Beattie, E. J., Jr., and Elias, W. S.: The Surgical Treatment of Intractable Asthma, J. Thoracic Surg. **20**: 596, 1950.
94. Gaensler, E. A.: Ventilatory Tests in Bronchial Asthma. Evaluation of Vital Capacity and Maximum Breathing Capacity, J. Allergy **21**: 232, 1950.
95. Wright, G. W., and Filley, G. F.: Pulmonary Fibrosis and Respiratory Function, Am. J. Med. **10**: 642, 1951.
96. Gordon, J., and Welles, E. S.: Decortication in Pulmonary Tuberculosis Including Studies of Respiratory Physiology, J. Thoracic Surg. **18**: 337, 1949.
97. Wright, G. W., Yee, L. B., Filley, G. F., and Stranahan, A.: Physiologic Observations Concerning Decortication of the Lung, J. Thoracic Surg. **18**: 372, 1949.
98. Carroll, D., McClement, J., Himmelstein, A., and Cournand, A.: Pulmonary Function Following Decortication of the Lung, Am. Rev. Tubere. **63**: 231, 1951.
99. Forsee, J. H., Kylar, S. L., and Blake, H. A.: Pulmonary Function in Traumatic Hemothorax Treated by Decortication, J. Thoracic Surg. **22**: 35, 1951.
100. Patton, W. E., Watson, T. R., Jr., and Gaensler, E. A.: Pulmonary Function Before and at Intervals After Surgical Decortication of the Lung, Surg., Gynec. & Obst., September, 1952.
101. Gaensler, E. A.: Air Velocity Index: A Numerical Expression of the Functionally Effective Portion of Ventilation, Am. Rev. Tuberc. **62**: 17, 1950.
102. Baldwin, E. de F., Harden, A., Greene, D. G., Cournand, A., and Richards, D. W., Jr.: Pulmonary Insufficiency. IV. A Study of 16 Cases of Large Pulmonary Air Cysts or Bullae, Medicine **29**: 169, 1950.
103. Long, J. H., Norris, C. M., Burnett, W. E., and Wester, M. R.: Bronchospirometric Studies in Bronchiectasis Before and After Lobectomy, J. Thoracic Surg. **19**: 477, 1950.
104. Pinner, M.: Pulmonary Tuberculosis in the Adult, Springfield, 1945, Charles C Thomas, p. 407.
105. Vaccarezza, R. F., and Soubrie, A.: Bronchospirometric Studies in Collapse Therapy; Functional Examination of Each Lung Before and After Establishment of Pneumothorax, An. Cated. de pat. y clin. tubere. **6**: 5, 1944.
106. Leiner, G. C.: Spirometric and Bronchospirometric Studies in Pneumothorax, Am. Rev. Tubere. **50**: 267, 1944.
107. Bruce, T.: Bronchospirometric Study of Lung Function After Concluded Pneumothorax, Acta Tubere. Scandinav. **20**: 68, 1946.
108. Marra, A.: La broncospirometria, Arch. di Tisiol. Napoli **5**: 423, 1950.
109. Vaccarezza, R. F., Soubrie, A., and Rey, J. C.: Estudios broncoespirometricos en la colapsoterapia. III. Examen funcional de los pulmones separado antes y despues de intituido el neumotorax extrapleural, An. Cated. de pat. y clin. tuberc. **9**: 48, 1947.

110. Gaensler, E. A., and Strieder, J. W.: Pulmonary Function Before and After Extrapleural Pneumothorax. A Comparison With Other Forms of Collapse and Resection, J. Thoracic Surg. **20**: 774, 1950.
111. Dressler, S. H., Bronfin, G. J., and Grow, J. E.: Pulmonary Function Before and After Extrapleural Pneumonolysis With Plombage, J. Thoracic Surg. **19**: 938, June, 1950.
112. Vaccarezza, R. F., Soubrie, A., and Rey, J. C.: Bronchospirometric Studies in Collapse Therapy: Functional Examination of Each Lung Before and After Induced Phrenic Paralysis Cases, An. Cated. de pat. y clin. tuberc. **8**: 165, 1948.
113. Leiner, G. C.: Spirometric and Bronchospirometric Studies in Thoracoplasty, Am. Rev. Tuberc. 53: 195, 1946.
114. Watson, T. R., Jr., and Gaensler, E. A.: Immediate and Late Respiratory Impairment Due to Selective Staged Thoracoplasty, Extraperiosteal Plombage and Extrapleural Conversion Thoracoplasty, Nat. Tuberc. A. Tr. **48**: 1952.
115. Kay, E. B., Perkins, H. M., and Crastnopol, P.: The Fate of the Residual Lobe Following Previous Lobectomy, Nat. Tuberc. A. Tr. **47**: 194, 1951.
116. Burnett, W. E., Long, J. H., Norris, C., Rosemond, G. P., and Wester, M. R.: The Effect of Pneumonectomy on Pulmonary Function, J. Thoracic Surg. **18**: 569, 1949.
117. Denolin, H., Duprez, A., and Callebaut, C.: L'exploration fonctionnelle des poumons séparés, Acta clin. belg. **4**: 516, 1949.
118. Paul, J. S., Beattie, E. J., and Blades, B.: Lung Function Studies in Poudrage Treatment of Recurrent Spontaneous Pneumothorax, J. Thoracic Surg. **22**: 52, 1951.
119. Gaensler, E. A.: Analysis and Critique of Pulmonary Function Studies, Bull. New England Med. Center **13**: 49, 1951.
120. Leiner, G. C., and Liebler, J. B.: The Technique of Bronchospirometry, Dis. Chest **17**: 578, 1950.
121. Steele, J. D.: The Practical Application of Bronchospirometry, Am. Rev. Tuberc. **47**: 608, 1943.

BRONCHOSPIROMETRY

II. Experimental Observations and Theoretical Considerations of Resistance Breathing

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IN A previous communication the literature on bronchospirometry was reviewed, and various catheters available for split function studies were described and compared.[1] Further reports concerning clinical and technical aspects of bronchospirometry are planned. During the course of 724 differential functional examinations, we felt that resistance breathing was the single most important factor in causing unphysiologic conditions during the procedure. The present study consists of an experimental and theoretical evaluation of resistance breathing and its relation to clinical experience.

Patients undergoing bronchospirometry are not under basal conditions. Discomfort due to the tube, apprehension, medication, topical anesthesia, accumulation of secretions, and spirometer inertia plus resistance all contribute to alterations in the metabolic rate, cardiac output, and ventilation. Because of the unusual breathing conditions, patients commonly hyperventilate. Metabolic changes and hyperventilation can be minimized by careful instructions to the patient, gentle technique of intubation, a quiet, orderly system in the operating room, the use of low resistance respiratory valves and well-balanced spirometers. However, even under these circumstances the establishment of a dependable physiologic base line is not possible. Therefore, in the interpretation of a single bronchospirometric study only the percentage of function of each lung relative to total function can be considered significant.[1]

A slight elevation of the metabolic rate and minute ventilation affect the lungs no more than slight exercise, and each lung contributes additional oxygen uptake and ventilation in the ratio of its functional capacity.[2] Two other factors, of greater importance, provide an abnormal background for both lungs during bronchospirometry, and also have been suspected of affecting one lung more than the other. The first factor is encountered in breathing a gas containing more oxygen than room air, which is necessitated by the technique of closed spirometry. This has been discussed and will be the subject of analysis in a future paper of this series.[3] The second factor is "resistance breathing."

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Resistance breathing, as the term is used here, implies any abnormal reduction of the lumen of the airway. This has been repeatedly mentioned as one of the problems during bronchospirometry, though its specific effects have never been investigated, either from a clinical or experimental standpoint.[1] There is no doubt that resistance breathing was marked with the early flexible double lumen catheters by Gebauer[4] and Zavod.[5] It was chiefly this difficulty which led to the Wright,[6] Carlens,[7] and Norris[8] modifications of these tubes.

Several problems must be solved before the specific effects of resistance breathing during bronchospirometry can be fully evaluated:

(A) Actual resistance of the several catheters to air flow at various speeds must be measured and these values compared with the resistance offered by the normal and abnormal airways of man.

(B) The type of flow within the various tubes, whether laminar (streamlined) or turbulent, must be determined. This would permit comparison between the type of air flow within the tubes and that occurring in the human respiratory passages.

(C) The effect of resistance breathing on the functional residual capacity must be examined.

(D) The pneumotachogram curve, representing instantaneous air flow, should be recorded of subjects breathing with and without the various tubes because this pattern is very sensitive to slight increases in resistance.

(E) Clinical experiences with the various tubes should be compared with special reference to the relationship of the tube used and the quality of the tracing and incidence of dyspnea during the test.

Vital capacity measured during bronchospirometry with the various tubes should be compared with values obtained during ordinary spirometry.

Finally, bronchospirograms obtained from the same patients but with different catheters should be compared.

(A) RESISTANCE TO AIR FLOW

To move a gas through a pipe it is necessary to create a difference in pressure between the two ends. This may be done by raising the pressure at one end above atmospheric (blowing) or by lowering the pressure at one end below atmospheric (sucking). The gas then flows from the opening at higher pressure to the one at lower pressure and suffers a loss of pressure equal to the pressure difference between the two ends of the tube. This loss of pressure, or "pressure drop," represents the energy expended in overcoming the friction created by the shearing forces of the molecular layers of the moving gas.

Method.—A steady flow of compressed air (Fig. 1) was passed through a rotameter (*A*), graduated in liters per minute. This instrument measures the volumetric rate of flow, much like the crude steel ball rotameter used on oxygen gauges and anesthesia machines. From the rotameter the gas passed into a large flask (*B*), provided with two further openings. The external orifice of the bronchospirometric catheter under investigation (*D*) was attached to one of these openings and an open-end water manometer (*C*) of the kind used in pneumothorax machines was attached to the other opening of the flask. The pressure in the flask, which was the pressure applied to the external end of the tube, was recorded by

the manometer. Since both the distal end of the catheter and the open end of the manometer were at atmospheric pressure, the manometer reading represented the "pressure drop" of the air flowing at the rate indicated by the rotameter. The rate of flow was adjusted for a reading of 5 liters per minute and the pressure drop was recorded. The flow was then increased in steps of 5 liters up to 40 liters per minute, and pressures were noted for each reading. Thus it was possible to measure the loss of pressure, or energy, required to move air through the various bronchospirometric tubes.

This technique was applied to each side of the two-lumen catheters. A different method was devised for the single-lumen Norris tube. Pressure drop was measured, as outlined, for the tube, which constitutes the airway for the left lung. To show the pressure drop at various rates of flow for the right lung, a trachea, together with vocal cords and surrounding tissue, was removed from a cadaver of a young man, and the Norris tube was placed through the cords into the trachea to this specimen. The tube was occluded internally with a piece of rubber, and the air outlet from the flask was attached to the lower end of the trachea.

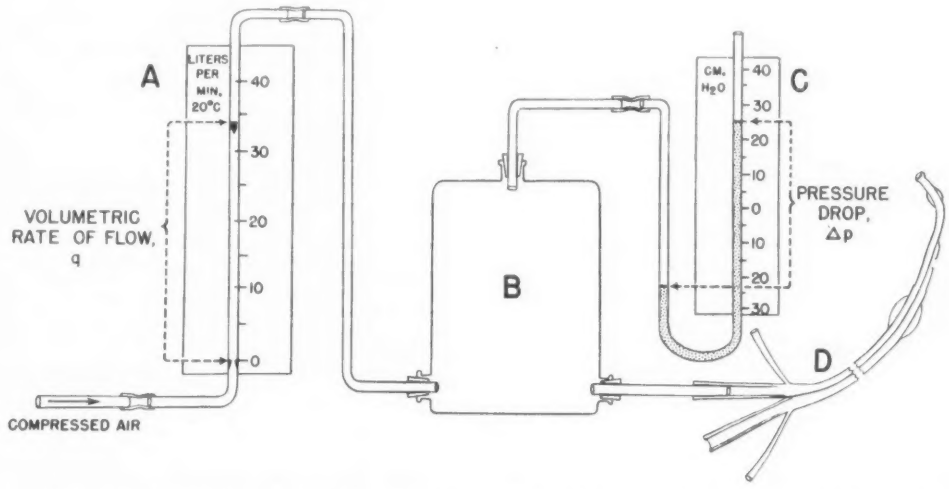

Fig. 1.—Schematic diagram of apparatus used for determination of pressure drop, Δ p, at various flow rates, q. Compressed air passes from a rotameter (*A*) which records the rate of flow, into a large bottle (*B*) for uniform distribution of pressure. The pressure drop or resistance is read from the water manometer (*C*) as the air passes out against the resistance of the catheter (*D*) to be tested.

The pressure drop measured was essentially the one due to the orifice between the relaxed vocal cords, partially occluded by the tube. To duplicate the most ideal conditions possible in the living, the experiment was repeated after a number of silk sutures had been placed through the glottis and pharynx and anchored to the edge of the cords. An even tension of 500 Gm. was then applied to every suture to pull the cords apart as much as possible. Pressure drop readings were then repeated.

The rates of flow used in the experiment were chosen to duplicate those encountered in clinical bronchospirometry. Silverman and associates[9] have shown that the highest flow rates occur during inspiration and that the peak inspiratory velocity is about 3.3 times the minute ventilation. The total minute ventilation, during our experience with bronchospirometry, ranged from 4.2 to 14.5 liters per minute. The peak rates of flow encountered in one limb of the bronchospirometric catheter were then 13.9 to 47.9 liters per minute if one lung was responsible for all the ventilation and the other lung was functionless. If both lungs participated equally the peak flow rates were 6.9 to 29.9 liters per minute. The experimental rates of flow which we have used, therefore, cover the range encountered during clinical bronchospirometry.

Results.—The pressure drop due to the flask and connecting tubing alone was established first. This was very small, ranging from zero at a flow rate of 5 liters per minute to 1.8 cm. of water at 40 liters per minute. These control pressures were deducted from all subsequent readings.

The pressure drop, Δ p, in cm. of water for the large (or male) size of the various catheters at volumetric rates of flow, q, from zero to 40 liters per minute are shown in Fig. 2. The pressure drop, or resistance, of the left airway was greater than that on the right in all of the two-lumen catheters to some extent

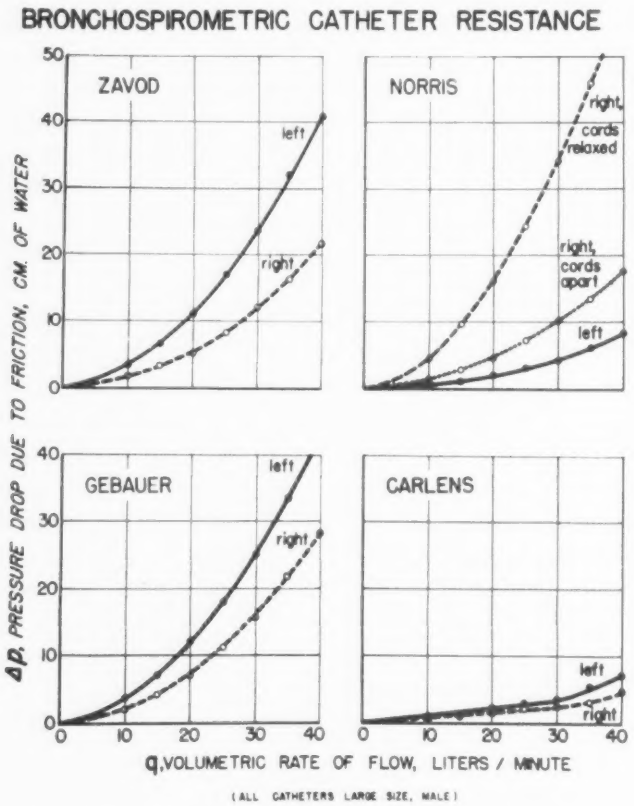

Fig. 2.—Pressure-flow diagrams for the various bronchospirometric catheters under the experimental conditions described.

because the tube to the left lung is always longer. At a flow rate of 40 liters per minute it was 22 cm. on the right and 41 cm. on the left for the Zavod catheter, 28 and 43 cm., respectively, for the Gebauer, and 5 to 7 cm. for the Carlens tube.

The resistance of the Norris catheter (airway to the left lung) was within the low range of the Carlens tube resistances. The resistance of the airway to the right lung with the vocal cords relaxed was 58 cm. of water at 40 liters per minute flow, the largest pressure drop recorded. The resistance to flow to the

right lung was over three times that recorded for the left at all measured flow rates. Maximal retraction of the cords lowered resistance considerably. Even so, the pressure drop was more than twice the resistance on the left.

Discussion.—The shape of the pressure-flow curve, the differences between the resistance on the right and on the left, and the magnitude of the actual resistance require further comment.

It is apparent from Fig. 2 that the relationship between the rate of flow and the resistance is not linear, except for flow from 5 to 30 liters per minute in the Carlens catheter, and from 5 to 20 liters for the left airway of the Norris tube. In engineering terminology, deviation from linearity means that the pressure drop does not represent the purely viscous resistance due to laminar flow alone, since during laminar flow the relationship between pressure drop and rate of flow is linear. The addition of a turbulent element is shown by the deviation of these curves from a straight line. From the practical standpoint of bronchospirometry, this means that even if the resistance of the two sides were the same for the same flow rates (which it is not), the lung ventilating a larger share of the total is more handicapped by the resistance factor than the diseased lung, since the increment of resistance, as the flow rate increases, is disproportionately greater than the increment of the rate of flow. This factor imposes an unequal burden on the two lungs which is not in proportion, but rather in inverse proportion to their ratio of ventilatory function. The effect is negligible with the Carlens catheter and at relatively slow rates of flow. It must be of considerable importance with use of the three other catheters, particularly at the highest flow rates.

Furthermore, the resistance of the two sides is not the same, even at the same rates of flow (Fig. 2). This causes a further handicap for the two lungs which is not in proportion to their relative ventilation. Again, for practical purposes, this problem has been solved in the Carlens catheter. The difference is of considerable magnitude in the other two-lumen catheters, and may, under certain circumstances, be most severe with use of the Norris tube (Fig. 2).

The experimentally determined range of the pressure gradients across the catheters can best be brought into its proper perspective by comparing it with that encountered in the normal and abnormal airways of man. When measured by the intermittent flow interruption technique, the pressure gradient or resistance, from mouth to alveoli in normal volunteers, ranged from 1 cm. of water at a rate of flow of 30 liters per minute to 4.3 cm. at 90 liters.[10, 11] Proctor and associates reported an average resistance of 1.9 cm. of water at a flow rate of 30 liters per minute in thirty-three determinations on normal individuals.[12] Readings ranged from 1.5 to 3.8 cm. In forty-seven patients with severe asthma and emphysema the same measurements were never greater than four times those observed in normal individuals. The highest resistance, recorded at 30 liters per minute, was 8 centimeters. The pressure drops quoted for the various catheters are therefore four to eighteen times greater than those occurring in the normal human respiratory passages. They are at least equal to, and may be four times greater than, the gradients encountered in the airways of patients with severe asthma or emphysema.

(B) TYPE OF FLOW WITHIN BRONCHOSPIROMETRIC CATHETERS

Some investigators have thought that the type of air flow encountered during bronchospirometry might be different from the kind of flow normally occurring in the respiratory passages.[8, 13] When movement of a fluid through a tube occurs as a telescopic sliding of adjacent concentric layers without transverse mixing, it is spoken of as streamlined or *laminar*. If erratic mixing of the concentric layers occurs, the flow is termed *turbulent*. If the kind of flow during bronchospirometry were different for the two lungs it would be of more than theoretical importance. When the flow becomes turbulent, the resistance sharply increases, since friction is no longer the result of intermolecular forces alone, but is augmented by the new internal shearing surfaces created by the eddying within the fluid. The point at which laminar flow becomes turbulent within the catheters can be determined from our experimental data or from theoretical considerations.

Type of Flow from Experimental Data.—The relationships between pressure drop and various factors affecting it during laminar flow were determined by Hagen[14] and Poiseuille[15] and can be written in the general form

$$\Delta p = \frac{L\ V\ \mu}{D^2\ \rho} \qquad (1)$$

where Δp is the pressure drop due to friction, μ is the viscosity of the fluid, L the length of the tube, V the linear velocity, ρ the density of the fluid, and D the diameter of the tube. For the same tube and the same gas at the same temperature the equation can be written

$$\Delta p = KV \qquad (2)$$

from which it is apparent that the relationship between pressure drop and velocity in laminar flow is linear.

From inspection of Fig. 2 it is seen that the flow is not laminar at any of the examined flow rates in the Zavod and Gebauer tubes and that it is laminar only at low rates of flow in the Carlens tube and the single lumen of the Norris catheter.

The pressure drop for *turbulent* flow is given by the Fanning equation.[16]

$$\Delta p = \frac{2\ f\ V^2\ L}{g\ D} \qquad (3)$$

where V, L, g, and D are the linear ve'ocity, length of tube, acceleration due to gravity, and tube diameter, as in equation (1), and f is a dimensionless friction factor. Again, as in the form (2), for the same tube and the same gas this can be written

$$\Delta p = K' V^2 \qquad (4)$$

In actual practice the exponent of V equals 1.0 in laminar flow. In turbulent flow the exponent is usually written as 2, although from experimental data it equals approximately 1.8.[17] The pressure drop and the velocity are here related in exponential or logarithmic fashion.

In order to investigate further the relationship between rate of flow and pressure drop, the experimental data of Fig. 2 were plotted on logarithmic paper (Fig. 3). The nonlinear relationship of flow and pressure drop proved to be logarithmic. For determination of the exponent of V in the abbreviated Fanning equation (4), the slope of the lines of Fig. 3 were measured and the exponent was found to equal 1.82. Turbulent flow therefore existed

throughout the entire range of flow rates investigated for the Zavod and Gebauer catheters and for the airway to the right lung with the Norris tube under the experimental conditions described.

Type of Flow from Theoretical Considerations.—To check the experimental findings it was thought desirable to calculate the rate of flow at which laminar flow would change to turbulent for each type of catheter.

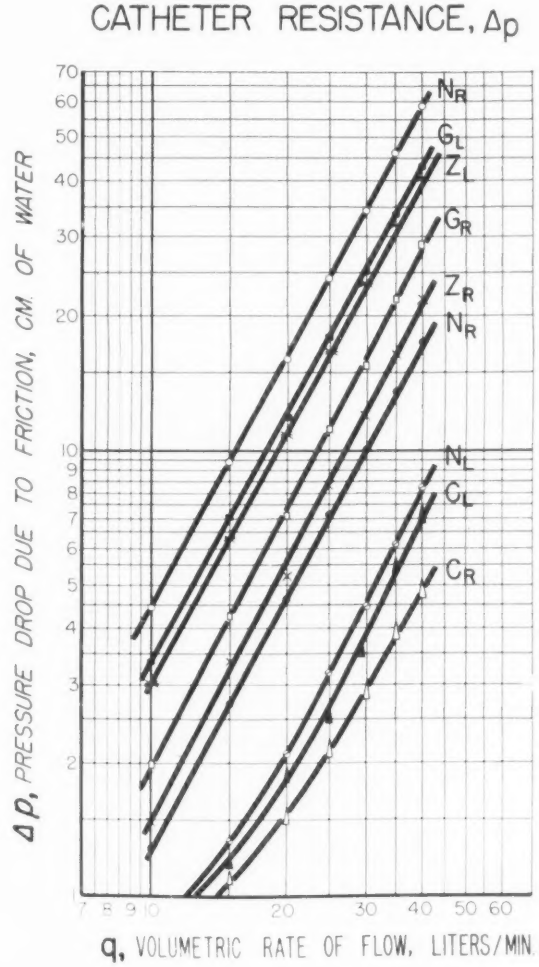

Fig. 3.—The pressure-flow curves of Fig. 2 plotted with logarithmic coordinates.

Reynolds[18] showed that the tendency to turbulence increases with increasing flow velocity, increasing diameter of tube and increasing density of fluid, and that it decreases with increasing viscosity of the fluid. He expressed this relationship in the form

$$R_e = \frac{V\ D\ \rho}{\mu} \qquad (5)$$

where R_e is the dimensionless Reynolds number, and ρ is the density, V the linear velocity, D the internal diameter of the tube and μ the viscosity as in previous equations. Since R_e is dimensionless it does not matter what units are used as long as such units are consistent.

Reynolds also found that as long as R_e remains below 2,000 the motion of the fluid is laminar. As R_e increases above 2,000 a brief transitional type of flow is followed by turbulence when R_e reaches 3,000. Knowing this, the critical velocity ($V_{Crit.}$), where laminar flow is lost, can be derived from equation (5)

$$V_{Crit.} = \frac{2{,}000\ \mu}{D\ \rho} \tag{6}$$

For air at 20° C. and 760 mm. Hg. pressure the viscosity, μ, is 0.0001827 Gm.-sec./cm.,[2] and the density, ρ, is 0.001246 Gm./c.c.[19] Therefore,

$$V_{Crit.} = \frac{2{,}000 \times 0.1466}{D} = \frac{293.2}{D} \tag{7}$$

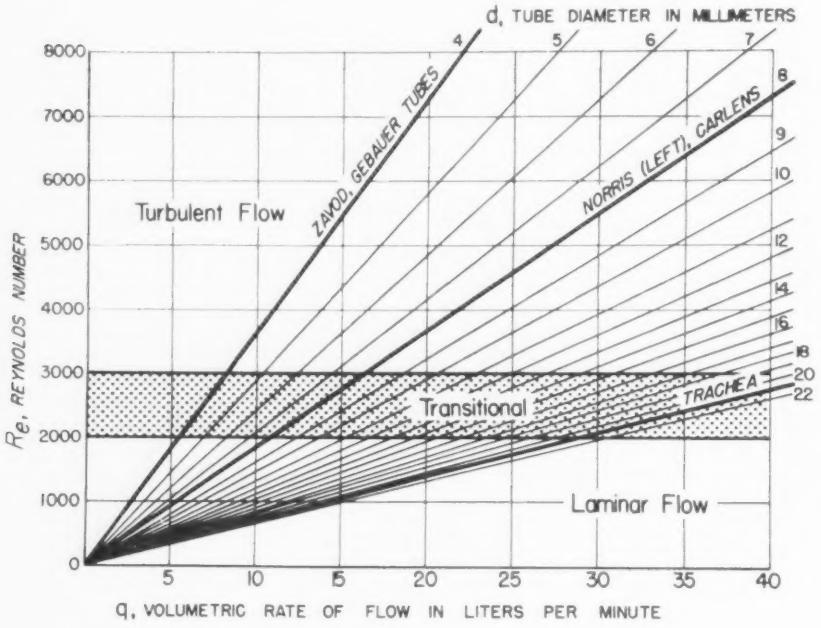

Fig. 4.—Relationship of the type of flow to the tube diameter, d, and the rate of flow, q, for air at 20° C. and 760 mm. Hg. Laminar flow occurs ordinarily for Reynolds numbers, R_e, of 2,000 or less, if the flow is steady and uniform.

For example, for the trachea, with an average internal diameter of 2.1 cm., $V_{Crit.}$ is about 140 cm. per second. The linear velocity, V, is equal to the volumetric rate of flow, q, divided by the cross-sectional area of the tube. With a tube of internal diameter D, the cross-sectional area is $\pi D^2/4$ and

$$V = \frac{q}{\pi\ D^2/4} \text{ or } q = V\ \pi\ D^2/4 \tag{8}$$

In our example, $q = 2.1^2 \times 140 \times \pi/4$ or 485 c.c./sec. Converting to minutes and liters the critical rate of flow for the trachea is 29 liters per minute.

Fig. 4 shows the calculation of the Reynolds number, R_e, for tubes from 4 to 22 mm. diameter at volumetric rates of flow, q, from 5 to 40 liters per minute for air at 20° C. and 760 mm. Hg. pressure. To find the critical rate of flow, ($q_{Crit.}$) for any given tube, it is necessary only to follow the horizontal line for $R_e = 2{,}000$, until it intersects the line for the desired tube diameter.

The calculated $q_{Crit.}$ for the 4 to 5 mm. tubes, the Zavod and the Gebauer catheters, as read from Fig. 4 is 5.1 liters per minute. Experimentally turbulence was encountered in these tubes at all flow rates above 5 liters (Fig. 3). The calculated $q_{Crit.}$ for the 7 mm. Norris and Zavod tubes is 10.2 liters (Fig. 4). Experimentally all flow rates above 10 liters caused turbulence in the Norris tube, and rates above 20 liters did the same for the Carlens tube (Fig. 2). Considering the fact that the internal diameter of the bronchospirometric tubes cannot be measured with any accuracy and that the diameter varies considerably in tubes made by the same manufacturer, the experimentally determined, and the calculated critical flow rates check very well.

The present discussion applies to experimental resistance studies. During clinical bronchospirometry, resistance is probably somewhat higher and turbulence occurs earlier since the equations for calculation of pressure drop (1), and (3), as well as for the Reynolds number (5) are valid only when the flow of the gas is *steady*, and *uniform*, when the tube is straight and has an end orifice and a smooth lining. None of these conditions is satisfied during actual use of the catheter. The mean flow rate changes during the respiratory cycle from a peak of 25 to 50 liters per minute to flow in the opposite direction of nearly equal magnitude, ten to twenty times each minute. The size and shape of the stream change constantly as the gas passes from the spirometer adapter into the catheter and from the catheter into the main-stem bronchi. Every one of these orifices creates turbulence. Even if the Reynolds number does fall below 2,000 at a given point, the orifice turbulence continues to disturb laminar flow for some distance beyond the obstruction.[20] Finally, during use, the tubes are not straight, the Gebauer and Zavod tubes do not have end orifices on the right, and the interior of the Norris tube is not smooth.

The same reasoning applies to a man breathing at rest without a bronchospirometric tube. Although the critical rate of flow of 29 liters per minute in the trachea may not be reached early in inspiration and expiration, this rate of flow is exceeded at the peak of every inspiratory and expiratory cycle. The turbulence created at that time is sufficient to disturb any tendency to laminar flow during the rest of the cycle. Furthermore, the narrow orifices of the nasal openings, the glottis and vocal cords, the many curves and angulations of the respiratory passages, and the relative roughness of their surfaces, all make the flow turbulent even during breathing at rest and without any catheter in place. From these studies it appears that the air flow is turbulent within all types of bronchospirometric tubes and under all types of functional distribution. This is the same type of air flow encountered normally during quiet breathing in the upper respiratory passage, in the trachea, and in the major bronchi of man. These conclusions are in contradiction to presently accepted ideas concerning the type of air flow encountered in the tracheobronchial tree of man at rest.[10, 12, 21]

Importance of the Tube Diameter.—The previously stated considerations emphasize the importance of the tube diameter. The significance of this single variable can be brought into proper perspective by substituting the volumetric rate of flow, q, for the linear velocity, V, in the equations for pressure drop (1) and (3), as was done previously for the $V_{Crit.}$ calculation. Since the linear velocity, V, equals the volumetric rate of flow, q, divided by the cross-sectional area of the tube of diameter D, Hagen-Poiseuille's law (2) becomes

$$p = \frac{K}{D^4} \tag{9}$$

and Fanning's equation (4) becomes

$$p = \frac{K'}{D^5} \tag{10}$$

These modified equations show that the pressure drop due to friction varies inversely as the *fourth power* of the diameter for laminar flow and as the *fifth power* of the diameter for turbulent flow. A very small diminution of the diameter therefore causes a relatively enormous increase in resistance to flow. This is borne out by clinical experience. The rela-

tively slight diminution in the diameter of the smallest respiratory passages due to the bronchial mucosal swelling and/or bronchospasm of asthma causes severe dyspnea and may be sufficient to cause death from ventilatory insufficiency. A small tumor impinging on, or protruding into, the trachea may not cause discomfort at rest, when maximal rates of flow are low, but may cause a severe limitation of exercise tolerance and of maximal ventilation.

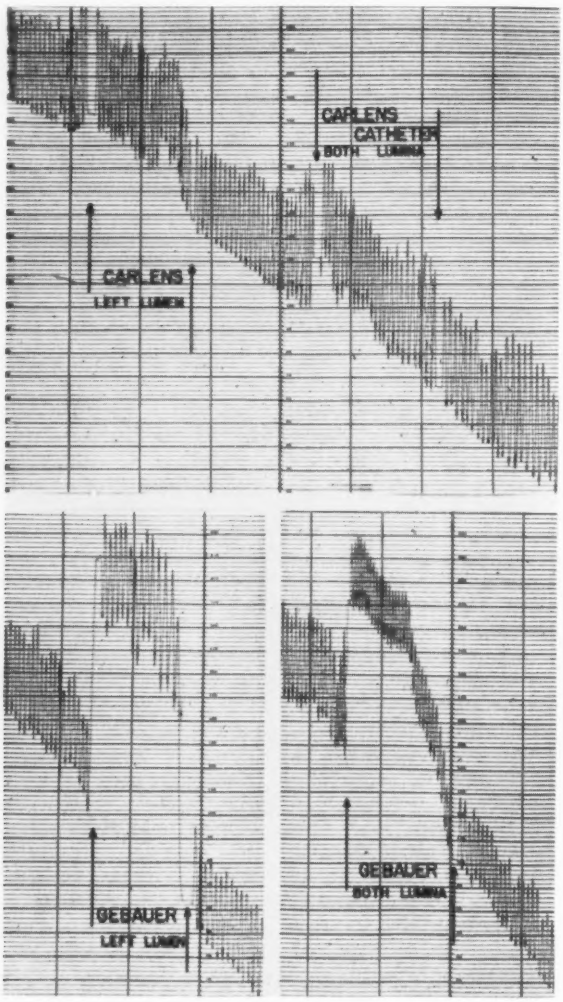

Fig. 5.—Spirograms of normal volunteers breathing alternatingly through large spirometer tubing and through various bronchospirometric catheters externally interposed in the breathing circuit.

(c) THE EFFECT OF RESISTANCE BREATHING ON THE FUNCTIONAL RESIDUAL VOLUME

Breathing with an added resistance has been shown to cause an increase in the functional residual volume[22] or an elevation of the pulmonary mid-position. This increase has been demonstrated irrespective of the site of the added resistance which may be located in the terminal bronchioles as in bronchial asthma,[23] in the major bronchi or trachea, or which has been added ex-

ternally in the form of a tracheotomy tube,[23] a gas-mask filter,[24] porous metal disks,[25] or thin glass tubes.[26] Whittenberger[23] has admirably summarized the compensatory mechanism responsible for this elevation of the functional residual volume: "As Laennec originally suggested, the response of functional residual volume to added airway resistance probably results from the fact that expiration is primarily a passive act. When resistance is added, the inspiratory muscles respond immediately to the increased work requirement by increased strength of contraction. If obstruction is produced just prior to a normal expiration, the potential energy of elasticity in the lung distended to that volume will not be sufficient to expel a normal tidal volume through the increased resistance in the time available before the next inspiration occurs.

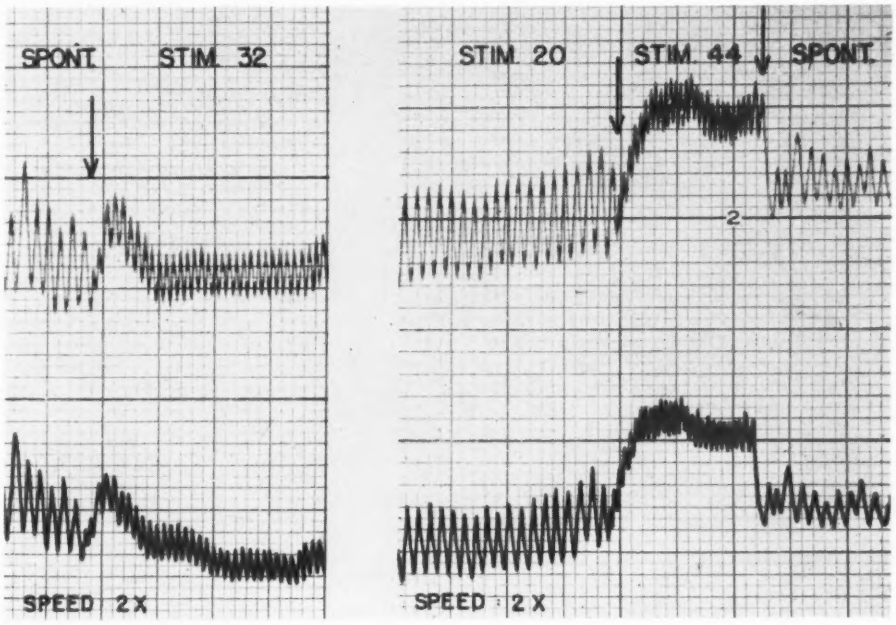

Fig. 6.—Bronchospirogram of a normal volunteer breathing spontaneously and at various increased rates induced by electrophrenic stimulation.

Since inspiratory volume is either normal or even greater than normal through a wide range of resistances, the lung volume will be greater than before at the end of the next inspiration. Under greater stretch, the lung has more energy available to expel the next tidal volume. Lung volume equilibration occurs at a point where the added pressure drop (resistance) is counterbalanced by the increased potential energy of the lung at the end of inspiration."

The increased work required for breathing against resistance has been shown to be accompanied by an increased oxygen requirement and by an increased cardiac output.[25] Resistance breathing must be in part responsible for the nonbasal values of cardiac output and oxygen uptake demonstrated during bronchospirometry.

Fig. 5 shows the spirographic tracings of a normal volunteer breathing, at times without significant resistance, and, at times, through an external resistance consisting of one or both lumina of various bronchospirometric catheters. Breathing through one, or simultaneously through both lumina of a Carlens tube, caused an elevation in the functional residual volume of only 200 to 300 milliliters. Breathing through both lumina of the Gebauer catheter caused an elevation of about 1,400 ml. and breathing through only one of the lumina elevated the functional residual volume by about 1,900 milliliters.

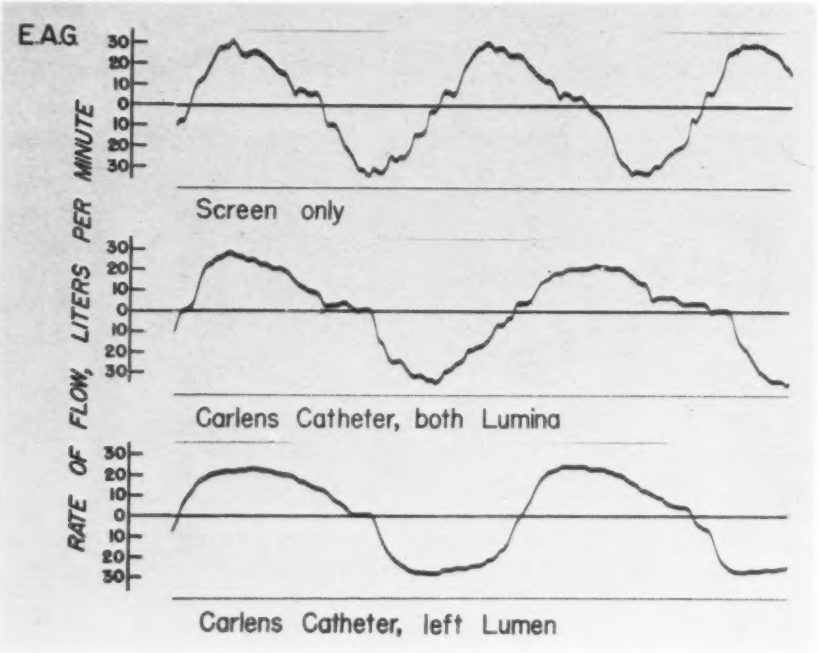

Fig. 7.—Pneumotachograms of a normal volunteer breathing without external resistance, and through one of both lumina of a Carlens catheter externally interposed.

This effect becomes more severe when an increase of respiratory rate causes a shortening of the time available for passive expiration. This is illustrated by bronchospirograms obtained during cyclical electrical stimulation of one phrenic nerve in man.[27] The first tracing of Fig. 6 suggests that the airway was adequate during spontaneous respiration. As electrical stimulation was started at the rate of 32 per minute, there was an initial elevation of the functional residual volume which reached a new equilibrium after a short period. The next tracing shows electrical stimulation at the rate of 20, with no apparent elevation. An increase in rate to 44 respirations per minute caused sustained elevation of about 500 ml. of the functional residual volume of each lung. There was a prompt return to the base line when electrical stimulations were discontinued.

(D) AIR FLOW PATTERNS AND RESISTANCE BREATHING

Instantaneous respiratory velocities can best be visually observed by pneumotachography as suggested by Fleisch.[28] Unlike the spirometer, which records

volumes of air moved, the pneumotachogram records the flow velocity of the air stream reflected in linear fashion by the pressure drop developed as the patient breathes through a fine Monel screen. In such a tracing the height of the curve at any given point indicates the rate of flow at that instant while the volume moved is expressed by the area underneath the curve. Inspirations are recorded below the zero line and expirations above it. The tracings of Figs. 7 and 8 were obtained with a pneumotachograph designed by Silverman.[29] The first tracing of Fig. 7 shows the air flow pattern of a normal volunteer.

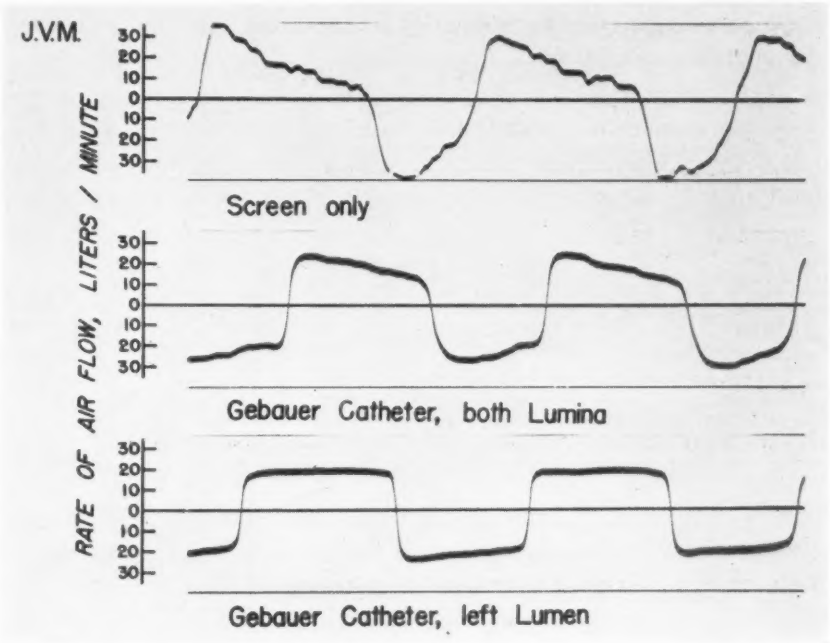

Fig. 8.—Pneumotachograms of a normal volunteer breathing without external resistance, and through one or both lumina of a Gebauer catheter externally interposed.

The middle tracing was obtained while breathing through an external resistance consisting of both lumina of a Carlens catheter. There was a slight blunting of the peak flow rates with no alteration in shape of the curve. The bottom tracing, obtained while breathing only through the left lumen of this catheter, shows further blunting of the peaks and obliteration of the air impulses due to changes in volume of the great vessels and heart. The top tracing of Fig. 8 shows the unobstructed pneumotachogram of another volunteer. Breathing through both lumina of the Gebauer catheter caused a complete loss of the volume pulsations transmitted from the heart and great vessels and a blunting of the peak inspiratory flow rates from about 45 to about 28 liters per minute. Breathing through only one lumen of the Gebauer catheter was not well tolerated by this subject and caused a sense of suffocation. The bottom tracing of Fig. 8 shows this pneumotachogram which is characteristic of severe resistance breathing.[23, 25] Both the inspiratory and the expiratory patterns have changed from an almost triangular shape to a nearly rectangular one. This was due

to a greatly decreased maximal flow rate, a decrease in the time required for reaching maximal flow rate and an increase in the total time required for an entire respiration.

(E) STATISTICAL DATA ON CLINICAL EXPERIENCE WITH THE VARIOUS BRONCHOSPIROMETRIC CATHETERS

The clinical observations during 724 bronchospirometries which are most closely related to resistance breathing are: the occurrence of dyspnea during the procedure, the quality of the tracings obtained, and the added bronchospirometric vital capacities as compared to the vital capacity previously obtained by ordinary spirometry.

We have always felt that dyspnea is not a contraindication to bronchospirometry. A number of patients who had bronchospirometry were therefore somewhat short of breath even before the procedure. The data summarized in Table I refer to the patients' report on dyspnea while the catheter was in place.

TABLE I. DYSPNEA DURING BRONCHOSPIROMETRY

CATHETER	NUMBER OF PATIENTS	NUMBER DYSPNEIC	PER CENT DYSPNEIC
Norris, with mouthpiece	20	6	30.0
Zavod	67	7	10.4
Gebauer, modified	548	40	7.3
Carlens	65	0	0.0
Total	700	53	7.6

Any marked increase in dyspnea could usually be recognized without questioning the patient. Of 700 patients in whom the catheter was successfully passed, 53 or 7.6 per cent showed an increase in shortness of breath during bronchospirometry. This never occurred with the Carlens tube and was encountered in about one-third of the patients with the Norris tube. The latter catheter invariably caused dyspnea when the right lung was responsible for a major share of function. In 67 bronchospirometries using the Zavod catheter, dyspnea was noted in 10.4 per cent of the studies and during 548 procedures with the modified Gebauer tube, dyspnea was noted in 7.3 per cent.

Except in uncooperative or frightened patients the quality of the tracing gave an indication of how severely the patients' respirations were disturbed by bronchospirometry. These data are related in Fig. 9. "Excellent," "good," and "fair" captions refer chiefly to regularity of breathing, and stability of the base line. One or more of the three available data of oxygen uptake, ventilation, and vital capacity could not be obtained when the tracing was "poor." No useful data were available from the tracings represented by the black area in Fig. 9. By far the largest number of "excellent" tracings were obtained with the Carlens tube while 54 per cent of the tracings with the Zavod and 31 per cent of the tracings with the Gebauer catheter were so classified. A complete set of data could be obtained from all tracings with the Carlens catheter, 89 per cent of the Gebauer tracings, and 90 per cent of the Zavod tracings.

One-fifth of the tracings obtained with the Norris single lumen tube was unsatisfactory. Satisfactory and complete data could be obtained from 90 per cent of all tracings.

It has been stated that the vital capacities from the left and right lung obtained during bronchospirometry should add up to 90 per cent of the previously obtained spirometric vital capacity.[30] Some use this percentage as a guide to proper placement of the catheter, and others regard the procedure as unsatisfactory if the added vital capacity is less than 75 per cent or more than 115 per cent of the spirometric vital capacity.[31] Carlens[7] performed ordinary

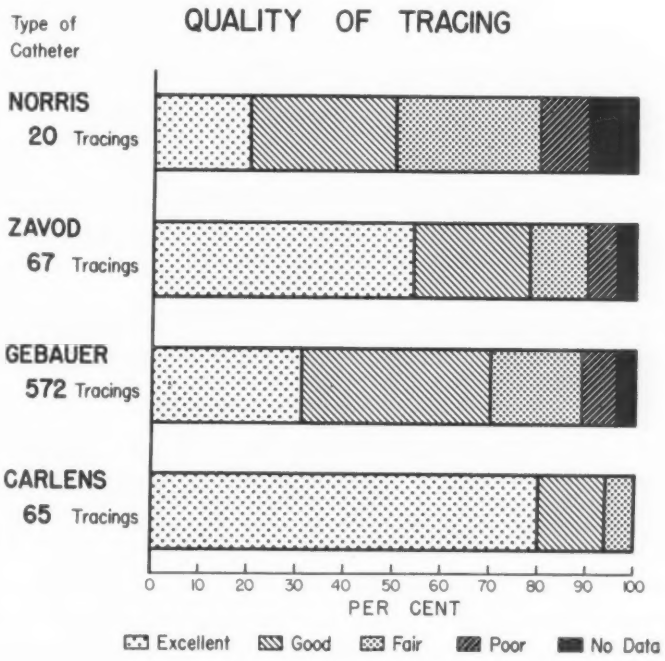

Fig. 9.—The quality of the bronchospirometric tracings as related to the use of various catheters. Classifications of "excellent," "good," and "fair" refer chiefly to regularity of breathing and stability of base line. One or two of the three obtainable data (oxygen uptake, ventilation, vital capacity) could not be read from "poor" tracings. The black areas indicate the percentage of tracings from which no useful data could be obtained.

spirometry one-half hour after bronchospirometry, and in this fashion surely handicapped his patients during spirometry. By this method he reported that the bronchospirometric vital capacity was 99 per cent of the total vital capacity with the use of his tube. We have had to compare the upright spirometric vital capacity with the supine bronchospirometric determination, since supine spirometry was not performed in every instance. However, if it is assumed that the upright vital capacity is about 10 per cent higher than the supine one the data presented on Table II can be corrected accordingly. The supine bronchospirometric vital capacity was 77 per cent of the upright vital capacity for the Zavod and modified Gebauer catheters. It was considerably better with the Carlens tube and poorer with the Norris tube (Table II).

TABLE II. SUPINE BRONCHOSPIROMETRIC VITAL CAPACITY COMPARED TO UPRIGHT SPIROMETRIC VITAL CAPACITY

CATHETER	NUMBER OF STUDIES	BRSP. V. C. AS PER CENT OF SPIR. V. C.
Norris, with mouthpiece	18	67
Zavod	60	77
Gebauer, modified	509	77
Carlens	65	88
Total	652	78

The bronchospirometric tracings of Fig. 10 were obtained in the same patients on consecutive days, initially with the Gebauer, and then with the Carlens catheter. The tracings illustrate all three points mentioned: dypsnea, quality of tracing, and vital capacity. Patient A was slightly dyspneic while the Gebauer tube was in place, and some doubt existed as to the accuracy of the tracing. The data obtained with the Carlens tube the next day showed identical ratios for the right and left lungs in every respect, but there was no dyspnea, the quality of the tracing had improved, and the vital capacity was now 96 per cent of the spirometric vital capacity. Patient B who had a functionless lung on the right was unable to ventilate properly through the left lumen of the Gebauer tube, and the procedure had to be interrupted because of severe dyspnea and cyanosis. No distress was noted the next day with the Carlens tube.

The clinical experiences parallel the data on resistance breathing obtained in the laboratory.

CONCLUSIONS

The Gebauer and Zavod flexible catheters presented a great advance over the Jacobaeus double-lumen bronchoscope. Many valuable data have been and will be collected with their help. Because of the great individual variation of these catheters, because of their inherently small internal diameter, and because of difficulties with proper placement, the procedure may under certain circumstances present considerable operative problems and results may be somewhat altered by aerodynamic inequalities.

The Carlens catheter signifies a further advance in bronchospirometry. Because of the ever-increasing use of this technique both for clinical evaluation of patients and for investigation of physiologic and physiopathologic problems, it is hoped that further advances in catheter design will be made.

SUMMARY

The resistances to air flow at volumetric flow rates from 5 to 40 liters per minute were measured for both lumina of the catheters by Gebauer, Zavod, Norris, and Carlens. They were four to eighteen times greater than the resistances encountered in the normal human respiratory passage and they were at least equal to, and at worst four times greater than, pressure gradients encountered in airways of patients with severe bronchial asthma or pulmonary emphysema. Unequal resistance of the two sides was shown to favor the right lung with the two lumen catheters, and the left with the one-lumen tube.

The shape of the pressure-flow curves showed a logarithmic relationship between rate of flow and resistance. It was shown that this favors the lung with impaired ventilation during bronchospirometry. The shape of the curves further suggested that, in the experimental conditions described, turbulence of

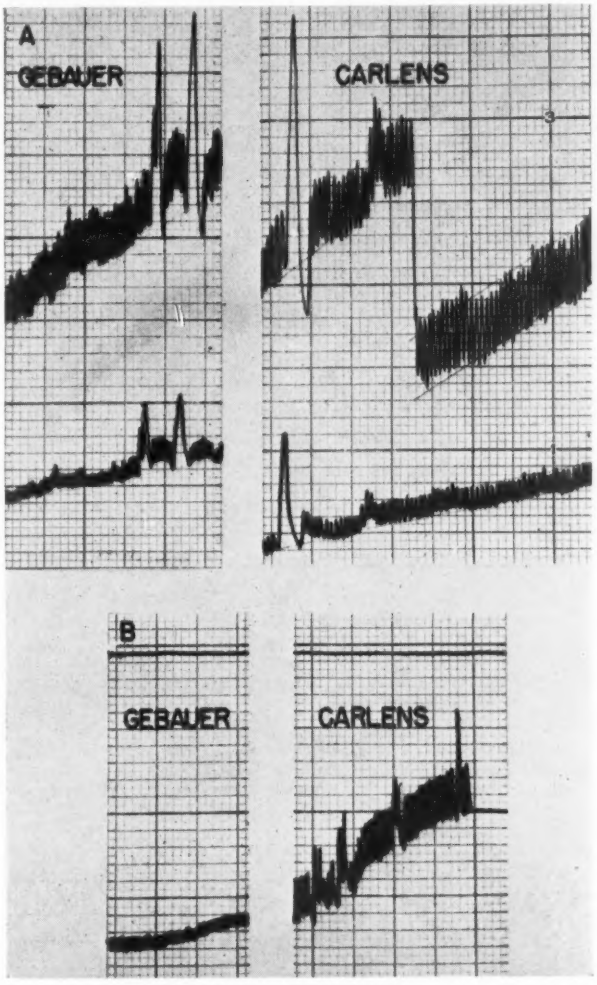

Fig. 10.—Bronchospirograms of the same individuals obtained on consecutive days with the Gebauer and the Carlens catheters. Patient A noted slight dyspnea with the Gebauer tube. Patient B, who had a functionless right lung, was unable to maintain ventilation through the Gebauer tube. The procedure was interrupted because of a respiratory distress and cyanosis.

flow occurred at all flow rates above 5 liters per minute in the Zavod and Gebauer catheters and in the airway to the right lung with use of the Norris tube. At the highest flow rates turbulence was encountered in all tubes.

The experimental results were supported by theoretical calculations. These calculations further suggested that turbulence prevails as well in the airways of normal men during quiet breathing at rest.

The importance of the internal diameter of the tubes is emphasized since the resistance varies inversely to the fifth power of the tube diameter if all other factors are kept constant.

The functional residual volume during bronchospirometry was shown to increase 200 to 300 ml. with use of the Carlens catheter. Other catheters caused an elevation up to 1,900 ml. in normal volunteers. The relationship of this effect to the respiratory rate was demonstrated.

Air flow patterns (pneumotachograms) were not significantly altered by the large diameter tubes. Patterns of severe resistance breathing were observed with other catheters.

Clinical experience during 724 bronchospirometries was related to the use of various catheters. Poor tracings were obtained in 20 per cent of twenty examinations with the Norris catheter, in 10 per cent of 67 procedures with the Zavod catheter, and in 11 per cent of 572 observations with the Gebauer catheter. No poor tracings were obtained during bronchospirometry with the Carlens catheter.

The average combined vital capacity during bronchospirometry was 67 per cent of the upright spirometric vital capacity using the Norris tube, 77 per cent with use of the Gebauer and Zavod tubes, and 88 per cent of the spirometric value with use of the Carlens tube.

Unusual subjective dyspnea was encountered during bronchospirometry in 30 per cent of operations with the Norris tube, 10.4 per cent with Zavod catheter, and 7.3 per cent with the Gebauer tube. Dyspnea was never encountered with use of the Carlens tube.

REFERENCES

1. Gaensler, E. A.: Bronchospirometry. I. Review of the Literature, J. Lab. & Clin. Med. **39**: 917, 1952.
2. Björkman, S., and Carlens, E.: The Lung Function During Rest and Exercise in Lung Diseases: A Bronchospirometric Study, Acta Med. Scandinav. Supp. **259**: 63, 1951.
3. Gaensler, E. A., and Cugell, D. W.: Bronchospirometry. IV. Ambient Air and Oxygen Recording Bronchospirometry. J. Lab. & Clin. Med. 1952.
4. Gebauer, P. W.: A Catheter for Bronchospirometry, J. Thoracic Surg. **8**: 674, 1939.
5. Zavod, W. A.: Bronchospirography. I. Description of the Catheter and the Technique of Intubation, J. Thoracic Surg. **10**: 27, 1940-41.
6. Wright, G. W., and Michelson, E.: Bronchospirometry, in Methods in Medical Research, Vol. II, Comroe, J. H. Jr., ed., Chicago, 1950, Year Book Publishers, Inc., pp. 82-90.
7. Carlens, E.: A New Flexible Double-Lumen Catheter for Bronchospirometry, J. Thoracic Surg. **18**: 742, 1949.
8. Norris, C. M., Long, J., and Oppenheimer, M. J.: Bronchospirography: Apparatus and Technique, J. Thoracic Surg. **17**: 357-365, 1948.
9. Silverman, L., Lee, G., Plotkin, R., Sawyers, L. A., and Yancey, A. R.: Air Flow Measurements on Human Subjects With and Without Respiratory Resistance at Several Work Rates, A. M. A. Arch. Indust. Hyg. & Occup. Med. **3**: 461, 1951.
10. Fenn, W. O.: Mechanics of Respiration, Am. J. Med. **10**: 77, 1951.
11. Otis, A. B., and Proctor, D. F.: The Measurement of Alveolar Pressure in Human Subjects, Am. J. Physiol. **152**: 106, 1948.
12. Proctor, D. F., Hardy, J. B., and McLean, R.: Studies of Respiratory Air Flow. II. Observations on Patients with Pulmonary Disease, Bull. Johns Hopkins Hosp. **86**: 255, 1950.
13. Proctor, D. F., McLean, R. L., and Lilienthal, J. L., in discussion on Wright, G. W., and Michelson, E.: Bronchospirometry, in Methods in Medical Research, Vol. II, Comroe, J. H. Jr., ed., Chicago, 1950, Year Book Publishers, Inc., pp. 90-91.
14. Hagen, G.: Über die Bewegung von Wasser in engen cylindrischen Rohren, Poggendorfs Ann. **46**: 423, 1839.

15. Poiseuille, J. M.: Recherches experimentales sur le mouvement des liquides dans les tubes tres-petits diameters, Ann. d. chim. et. d. phys. **7**: 50, 1843.
16. Walker, W. H., Lewis, W. K., McAdams, W. H., and Gilliland, E. R.: Principles of Chemical Engineering, New York, 1937, McGraw-Hill Book Co., Inc., pp. 40-99.
17. Prandtl, L., and Tietjens, O. G.: Applied Hydro- and Aeromechanics, New York, 1934, McGraw-Hill Book Co., Inc., pp. 14-57.
18. Reynolds, O.: An Experimental Investigation of the Circumstances which Determine whether the Motion of Water Will be Direct or Sinuous, and of the Law of Resistance in Parallel Channels, Phil. Trans. Roy. Soc. London, Sci. Papers **2**: 51, 1883.
19. Handbook of Chemistry and Physics, Hodgman, C. D., ed., 32nd ed., Cleveland, 1950, Chemical Rubber Pub. Co., pp. 1791, 1835.
20. Dodge, R. A., and Thompson, M. J.: Fluid Mechanics, New York, 1937, McGraw-Hill Co., Inc., pp. 192-233, 275-300.
21. Rohrer, F.: Der Strömungwiderstand in den menschlichen Atemwegen und der Einfluss der unregelmässigen Verzweigung des Bronchialsystems auf den Atmungsverlauf in verschiedenen Lungenbezirken, Arch. f. d. ges. Physiol. **165**: 419, 1916.
22. Standardization of Definitions and Symbols in Respiratory Physiology, Fed. Proc. **9**: 602, 1950.
23. Whittenberger, J. L.: Lung Volume and Air Flow Characteristics in Asthma, in Abramson, H. A.: Somatic and Psychiatric Treatment of Asthma, Baltimore, 1951, The Williams and Wilkins Company, pp. 50-61.
24. Bruns, O.: Veränderungen der äusseren und inneren Atmung bei Stenose der oberen Luftwege, Deutsch. Med. Wchnschr. **55**: 902, 1929.
25. Cain, C. C., and Otis, A. B.: Some Physiological Effects Resulting from Added Resistance to Respiration, J. Aviation Med. **20**: 149, 1949.
26. Thiel, K.: Über Mittellageveränderung durch Stenosierung der oberen Luftwege, Ztschr. f. d. ges. exper. Med. **67**: 810, 1929.
27. Sarnoff, S. J., Gaensler, E. A., and Maloney, J. V., Jr.: Electrophrenic Respiration. IV. Bronchospirometry During Stimulation of one Phrenic Nerve in Man, J. Thoracic Surg. **19**: 929, 1950.
28. Fleisch, A.: Der Pneumotachograph; ein Apparat zur Geschwindigkeitsregistrierung der Atemluft, Arch f. d. ges. Physiol. **209**: 713, 1925.
29. Silverman, L., and Whittenberger, J. L.: Clinical Pneumotachograph, in Methods in Medical Research, Vol. II, Comroe, J. H., Jr., ed., Chicago, 1950, The Year Book Publishers, Inc., pp. 104-112.
30. Leiner, G. C., and Liebler, J. B.: The Technique of Bronchospirometry, Dis. Chest **17**: 578, 1950.
31. Simonin, P., Girard, J., Sadoul, P., Grilliat, J. P., and Braun, P.: L'Exploration separees des poumons par bronchospirometrie; etude critique d'apree 80 separations, Rev. Med. de Nancy **75**: 250, 1950.

LABORATORY METHODS

A STANDARD ACTIVITY CURVE FOR ESTIMATION OF PROTHROMBIN USING THE ONE-STAGE METHOD

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THE determination of "prothrombin activity" of human blood has been the subject of much investigation, and yet it appears that no method thus far is wholly satisfactory.[1] This type of assay is an extremely complex reaction involving many factors.[2-9] Chief among the problems, particularly in the one-stage method, is the development of a stable thromboplastin. This has been attempted by many, and a considerable measure of success has been achieved.[1, 10-14] In laboratories in which sufficient time is devoted to enable the exact duplication of proposed methods, the tests are done well enough for practical purposes.

This report deals with a method of calculation conceived by one of us (J. H. T.) which has now been in use in this laboratory for two years and also in the clinical laboratories of the Indianapolis General Hospital with satisfactory results. During this period the usual method of estimating prothrombin activity of dicoumarolized and other patients' blood samples was applied using the ordinary or conventional activity curve. Each day the results were compared with those obtained by a new method of calculation. Quick,[15] in 1942, mentioned a method of calculation which is based on a somewhat similar reasoning. Our work, however, results from an observation that the *ratio of activity* when using a partially deteriorated and a fully active thromboplastin is essentially the same. In other words, a parallelism exists between curves produced with thromboplastins of different potencies. Practical application and demonstration of this fact is possible by simple conversion of the prothrombin coagulation *times* between a normal undiluted and diluted plasma to represent "relative per cent change" between these two times. This figure is then applied to a standard conversion curve to be described. The formula may be expressed:

Relative per cent change in prothrombin time (PT) in seconds equals

$$100 - \left(\frac{\text{control PT in seconds} \times 100}{\text{patient's PT in seconds}}\right)$$

The thromboplastins from various sources have exhibited more or less loss of potency after preparation, and even more disturbing was the slight but important variation of daily reconstituted dried commercial preparations[16] which required frequent construction of curves. Most of the work undertaken in this laboratory has been done with preparations of human placenta. On numerous occasions, however, we have compared products commercially available as well as those made in this laboratory according to the methods of Hurn, Barker, and Magath.[17]

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MATERIALS AND METHODS

1. *Thromboplastin.*—Human placental thromboplastin was prepared by saline extraction of fresh perfused placentas and subsequent purification by precipitation (pH 5.0 to 5.5), absorption with magnesium hydroxide and freeze drying after adding 5 per cent lactose and 1:10,000 Merthiolate (Thimerosal, Lilly). This material was divided into 2 ml. amounts and placed in 20 ml. bottles before drying. The bottles were stoppered immediately after drying in a room of low relative humidity, and were stored at 5° C. until used. The preparation was reconstituted with 2 ml. distilled water and allowed to remain at 5° C. for at least twelve hours before use.

2. *Deprothrombinized Pooled Plasma.*[18]—One hundred milligrams of powdered barium sulfate were added per milliliter of pooled plasma, mixed, incubated at 37° C. for ten minutes, and centrifuged at 2,200 r.p.m. for fifteen minutes. The clear supernatant plasma was tested with thromboplastin of maximum potency and calcium chloride solution to verify that the plasma had no prothrombin activity. This plasma then was used as a diluent for normal plasma in the construction of activity curves.

Preparation of all other solutions and plasmas and the technique of performing the test were carried out as described by Quick in the one-stage procedure,[10] with the exception of sodium oxalate, 1.34 per cent. This material was used in the usual proportions, exactly 1 ml. being introduced into a sterile, dry 10 ml. syringe, into which 9 ml. of the patient's blood was drawn by direct venipuncture. Partial mixture of the anticoagulant during collection of the blood sample thus reduced the possibility of even small fibrin formations, the presence of which resulted in false high prothrombin assays. The anticoagulant was stored in 1 and 5 ml. sealed autoclaved ampules. In our hands sterility of this material was found necessary for satisfactory control measures after early experience with unsterile sodium oxalate which occasionally resulted in false low prothrombin results.

Procedure.—Plasma from several (four to six) presumably normal individuals was obtained and pooled. Tests were performed to determine the time (in seconds) required for the clot formation, using the undiluted plasma, as well as the clotting time of 1:2, 1:4, and 1:8 dilutions. The dilutions were made with plasma treated with barium sulfate as described above. It is assumed that the normal, pooled, undiluted plasma contained 100 per cent "prothrombin activity," and in the dilutions proportionate amounts. A typical result is shown in Table I.

TABLE I

PLASMA DILUTION	THEORETICAL PER CENT PROTHROMBIN ACTIVITY	SECONDS
Undiluted	100	20
1:2	50	27.5
1:4	25	38.5
1:8	12.5	51

Preparation of Conversion Graph.—The following calculations are made, in the case of each dilution of plasma, for the purpose of converting the speed of the reaction to percentage reduction of prothrombin time between the normal clotting time and the clotting time of the diluted plasma. *Undiluted plasma = 20 seconds, which is equivalent to 100 per cent theoretical prothrombin activity (TPA). 1:2 dilution of plasma (50 per cent TPA) = 27.5 seconds, which is a 27.3 per cent relative change of prothrombin activity as compared to the normal. 1:4 dilution (25 per cent TPA) = 38.5 seconds, or 48.1 per cent relative change of prothrombin activity. 1:8 dilution (12.5 per cent TPA) = 51 seconds, or 60.8 per cent relative change of prothrombin activity.*

These percentages are obtained in each instance by dividing 20 (the number of seconds required for the prothrombin clotting time of the normal undiluted plasma) by the diluted normal plasma clotting time, in seconds, times 100, and subtracting from 100 per cent. It should be noted that this figure indicates "per cent change of activity" (reduction in speed of reaction) when 100 per cent (no reduction) represents the normal plasma clotting

time. Our curves are based on this particular type of calculation mainly for the sake of simplicity, e.g., Fig. 1, the 50 per cent (1:2) dilution of normal plasma results in a 27.3 per cent *relative change of activity* as compared to the normal, which could also be interpreted as a 37.5 *per cent increase of prothrombin clotting time*. Each figure applied to an appropriate conversion curve, however, results in 50 per cent prothrombin activity.

The values 0, 27.3, 48.1, and 60.8, representing the relative changes of the reactions at the various dilutions (in per cent), are plotted graphically against the relative prothrombin concentration of the diluted normal plasma. The reaction values are plotted on the abscissas against the per cent of prothrombin concentration on the ordinates.

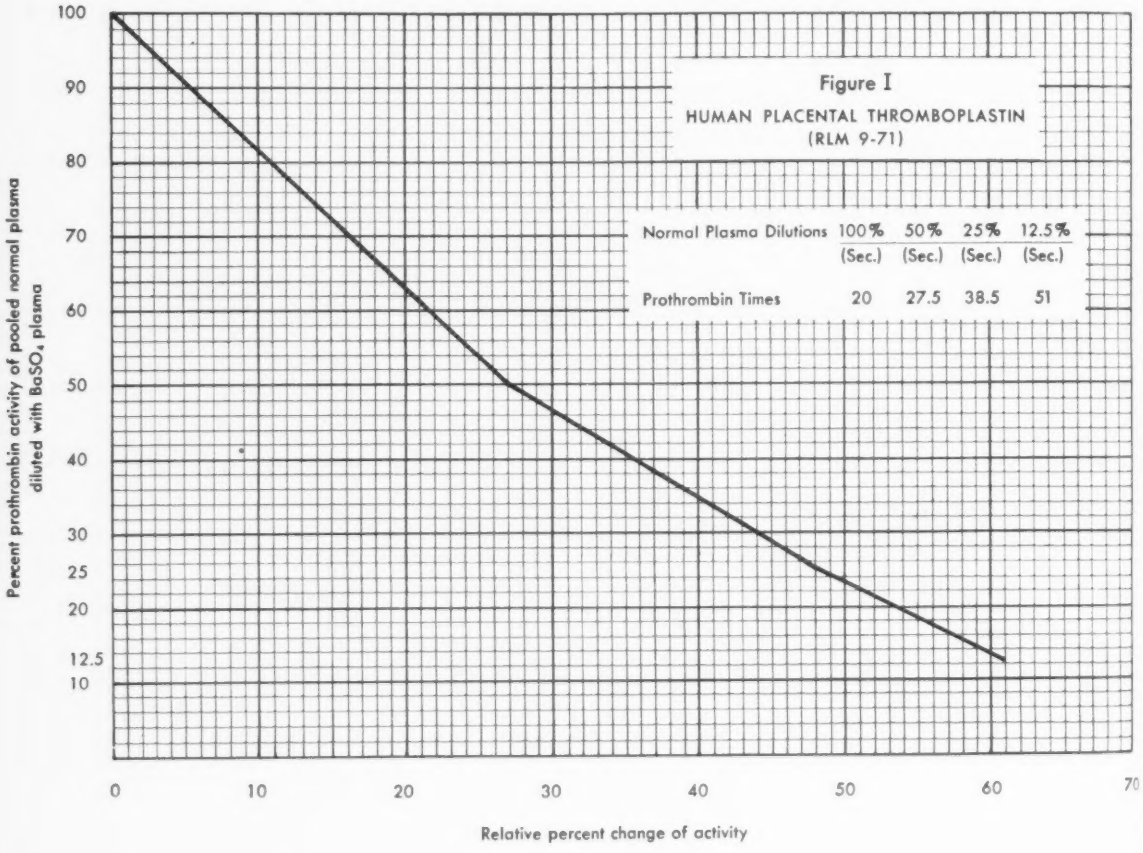


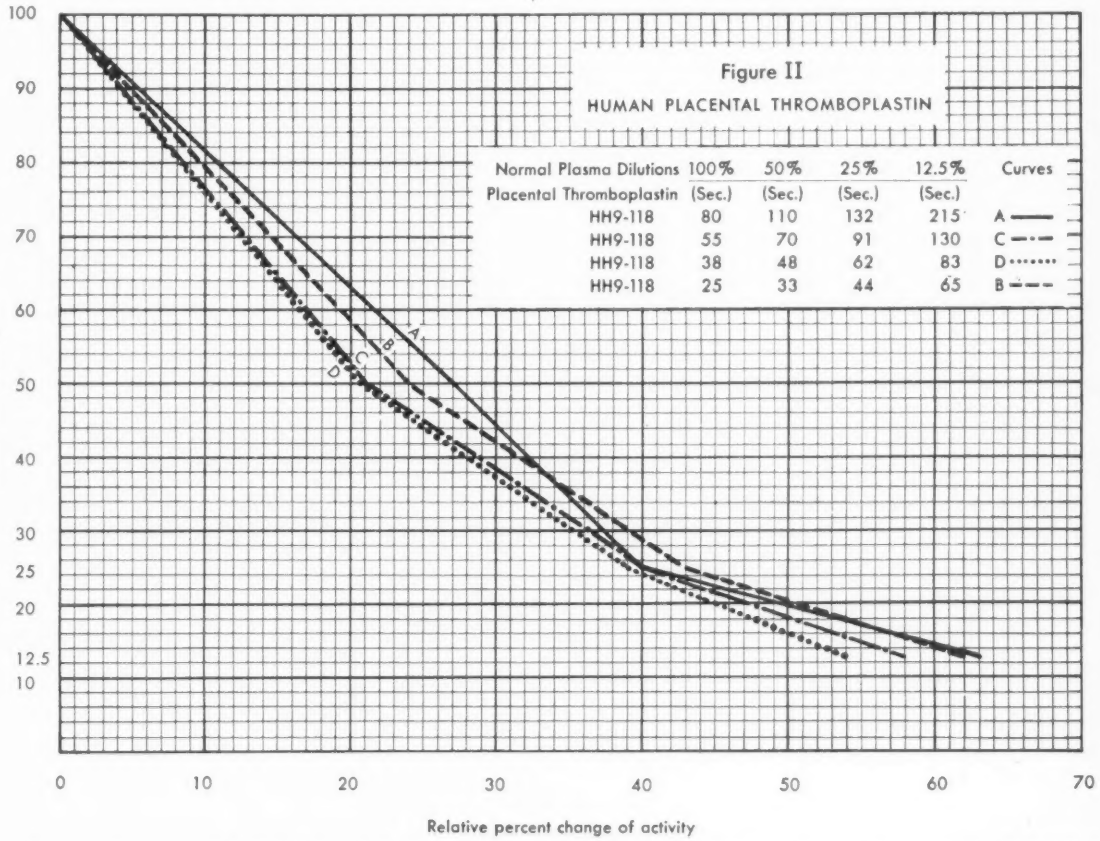
Fig. 1.

Following the construction of this curve (Fig. 1) for a given thromboplastin preparation, it is then only necessary to test two "normal" plasmas each day to determine the average time, in seconds, required for the thromboplastic preparation (even though it has lost some activity) to clot the undiluted normal plasma. Occasionally there was found to be more than a one-second difference between the two normals. In this event a third normal plasma was tested. The "unknown" plasma is similarly tested. These values are converted according to the foregoing formula, and the resulting figure is applied to the graph described in Fig. 1. Then, reading the corresponding point on the ordinate, the value of the unknown is determined as "per cent prothrombin activity."

Example: Normal control plasma prothrombin time (control PT) = 20 seconds. Patient's plasma prothrombin time (patient's PT) = 45 seconds. $100 - (20 \div 45 \times 100) = 56$ per cent relative change (or reduction) of prothrombin activity, which is equivalent to 18 per cent prothrombin activity.

Fig. 2 demonstrates clearly the close activity ratio of four conversion curves which were derived from an extremely wide variation of thromboplastic potencies using the same normal control plasma. Samples of thromboplastin were taken from the same original batch of material and allowed to deteriorate to different degrees after remaining at room temperature for varying lengths of time. It can be seen from this that marked loss of thromboplastic potency when compared to the fully potent material will not appreciably affect the final determination of prothrombin activity of a given plasma regardless of which of the four curves are used. This verifies the validity of the formula.

Table II shows comparative results from dicoumarolized patients' plasma obtained simultaneously using a fully potent and a partially deteriorated thromboplastin.



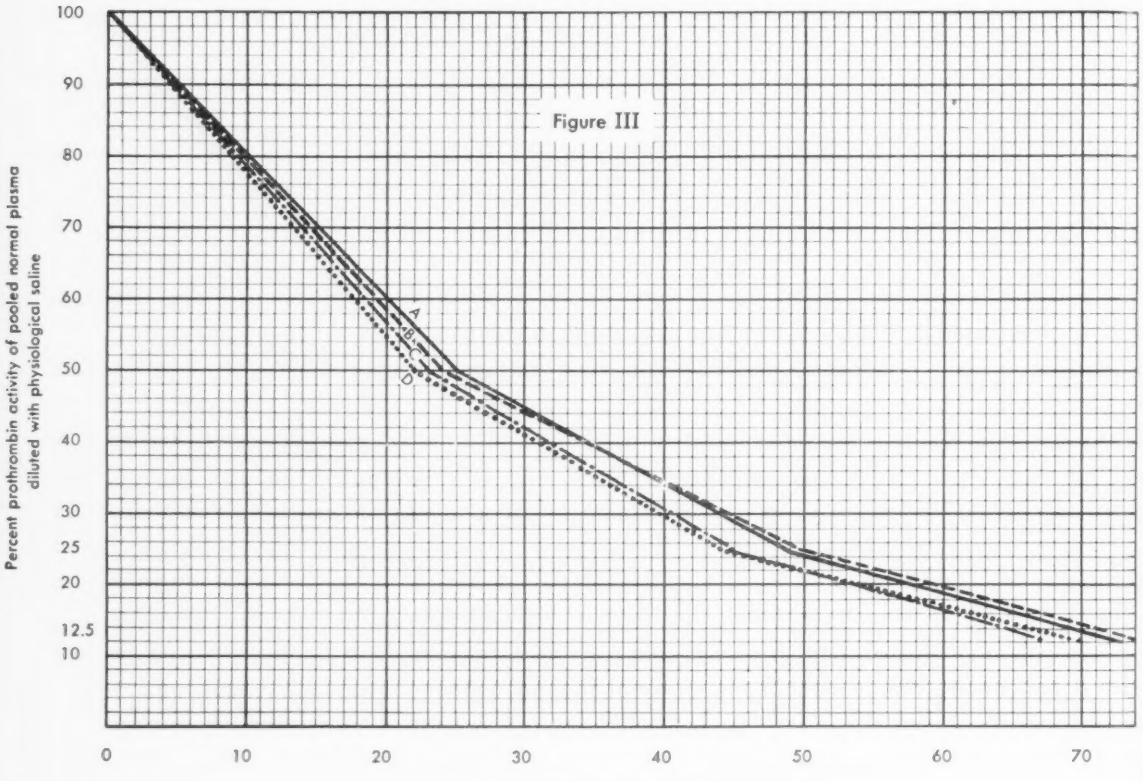
Four conversion curves derived simultaneously from serial dilutions of same pooled normal plasma using same lot preparation (HH9-118) of placental thromboplastin which was divided into four samples. Three of the samples were allowed to deteriorate at room temperature for two to nine days; the fourth sample (B) was kept refrigerated (5°C.) for control.

Fig. 2.

Fig. 3 shows the close relationship between our conversion curves derived from different ampules and batches of rabbit brain thromboplastin,* each of which had different potency on the same undiluted normal plasma. While the latter variation may be slight, it is obvious that use of the conventional type activity curve cannot be applied correctly for daily work unless an appropriate curve is used which coincides precisely with the normal plasma prothrombin time at the time of testing. One "standard conversion curve," we have found, obviates this difficulty and corrects for such differences in thromboplastic potency from day to day.

*Difco thromboplastin.

This method did not, of course, solve the problem of the normal control, which meant that the results from day to day were fully dependent upon obtaining normal control plasma. As a means of experimentally verifying the normalcy of these daily control fresh plasmas secured from different and supposedly normal individuals, we felt a standard or artificial control plasma would fulfill the requirements. Because we had been unable to depend on frozen stored normal plasma for this purpose, we found the following to be satisfactory.



Sample of conversion curves derived from different batches of Difco thromboplastin and constructed at different times during a six-month period. Included also is conversion curve D, which was interpolated from the pamphlet included with thromboplastin from Difco Laboratories.

Curve A.....Undiluted normal plasma—15.0 seconds
Curve B.....Undiluted normal plasma—17.5 seconds
Curve C.....Undiluted normal plasma—12.0 seconds
Curve D.....Undiluted normal plasma—12.5 seconds
(Interpolated from Difco Laboratories pamphlet. Control 403420)

Fig. 3.

Dried human plasma such as is used for plasma transfusions was reconstituted, 92 mg. in 1.0 ml. sterile distilled water. This plasma consistently clotted in forty-eight to fifty seconds with a fresh potent placental thromboplastin (normal plasma 26.5 seconds). If, then, the potency of the thromboplastin decreased by as little as 2 seconds, it would be reflected in the reconstituted plasma by delayed clotting times of at least 8 to 10 seconds (from 48 to 56 seconds) or considerably more. However, if the normal control fresh plasma produced clotting 2 or more seconds longer than the usual value (26.5 seconds) and reconstituted dried plasma was 48 to 50 seconds, we knew the fault was in the fresh control which was then

discarded. Simply then, a longer than 48 to 50 second prothrombin time with the reconstituted plasma meant that the thromboplastin was partially deteriorated. In addition the "relative per cent change of prothrombin time" of this material to a previously known normal, regardless of thromboplastic potency, was invariably between 45 to 47 per cent. Therefore, use of a dried human plasma with an unknown control that produced such a figure was evidence of the reasonable normalcy of the control. (The entire dried plasma transfusion unit was transferred to a wide mouthed jar with a screw lid and stored at 5° C. continuously for over twelve months. In other hands the prothrombin *time* of such plasma may be quite different from our figures. The essential point, however, is we found this material to give consistent values when small samples were reconstituted daily. Because of this we were able to use it in the manner described.)

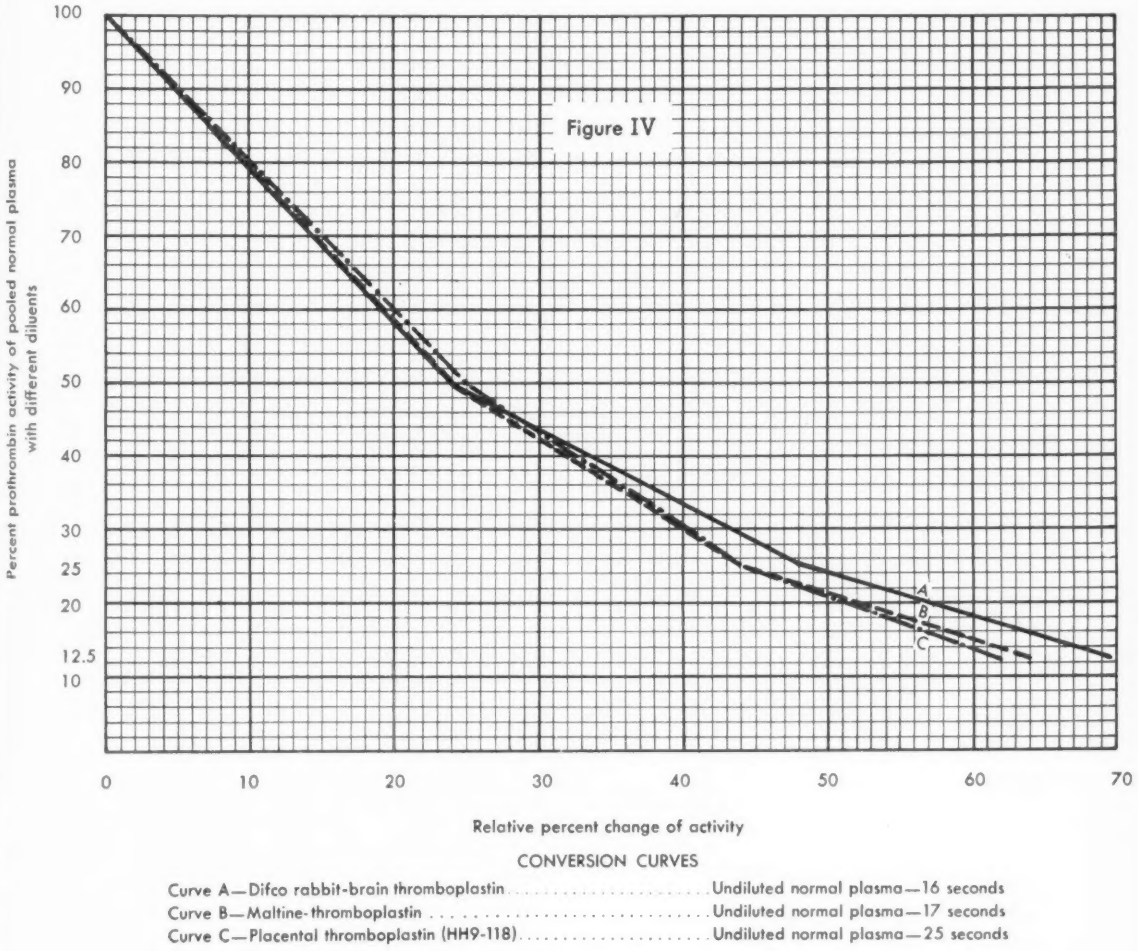


Fig. 4.

It should be emphasized that the foregoing was primarily an experimental procedure during the development of the new type curve. While useful, it was found unnecessary for routine work provided care was exercised in the selection of control plasmas presumed to be normal with respect to prothrombin. To obviate this two such normal plasmas were obtained each day and their average prothrombin clotting times used as the control figure, providing they coincided within one second of each other.

TABLE II

PATIENT NO.	PATIENTS' PROTHROMBIN ACTIVITY VIA THE "USUAL TYPE NORMAL" CURVE USING A FULLY POTENT PLACENTAL THROMBOPLASTIN (PER CENT)	NORMAL CONTROL PLASMA (SECONDS)	PATIENTS' PROTHROMBIN ACTIVITY VIA THE "CONVERSION CURVE" USING A DETERIORATED PLACENTAL THROMBOPLASTIN (PER CENT)	NORMAL CONTROL PLASMA (SECONDS)
1	42	26	46	55.5
2	35	22	43	45
3	13	26.5	14	37
4	64	26	56	30.5
5	16	26	17	30.5
6	34	26.5	26	36
7	33	33	36	59.5
8	41	32.5	47	39

DISCUSSION

The combination of the standard conversion curve and the fresh normal plasmas afforded a means to quickly and simply test our dicoumarolized patients' plasmas with considerably less chance of error than heretofore without the necessity of frequently constructing new curves of the conventional type.

The problem of dilution of normal plasma with either $BaSO_4$ deprothrombinized plasma or normal saline appears to be complex, and duplication of all such activity curves is not attainable. Conley and Morse[19] have shown these variations using different diluents, and our results are much in agreement.

With placental preparations, when 0.85 per cent sodium chloride is used to dilute the normal plasma serially, the pattern of the curve between the 100 per cent and 25 per cent dilution range is steeper than when $BaSO_4$ is the plasma diluent. When the latter is employed, prothrombin time below the 25 per cent dilution is longer than if the saline is used. However, this relationship is reversed when Difco rabbit brain thromboplastin is employed. We believe that saline probably dilutes *all* of the clotting factors in the normal plasma including prothrombin, while barium sulfate actually adsorbs and removes prothrombin, and in our hands has been found quite satisfactory. Provided the loss of other coagulation factors by such procedure is not deleterious in the one-stage test, the latter might be considered the more specific diluent.[19]

Upon comparing our conversion curves constructed from different thromboplastins and the two types of normal plasma diluents, we find close agreement between curves derived from: (1) Difco (rabbit brain) thromboplastin, using saline as diluent; (2) Maltine thromboplastin,† using $BaSO_4$ deprothrombinized plasma as diluent; and (3) placental thromboplastin, using $BaSO_4$ deprothrombinized plasma as diluent. The usual type curves, which we experimentally calculated to the conversion type curve, are shown in Fig. 4. In addition, when the normal type curves of Conley and Morse[19] were also recalculated on the basis of the standard conversion curve, they were found to be strikingly similar to those we constructed in this laboratory.

From the standpoint of the commercial thromboplastins we have had quite satisfactory results when using Difco rabbit brain preparations for routine work.

†Supplied in 50 mg. tablet form in sealed vials by the Maltine Company, Morris Plains, N. J.

This material has been reconstituted (2 to 4 ampules at one time) and used for as long as six weeks or more when stored at 5° C., 0.85 per cent sodium chloride solution as diluent being used for curve construction. The slight loss of thromboplastic potency upon such storage was automatically corrected for by use of the standard conversion curve. While it may be desirable that a new conversion curve be prepared from each new lot of thromboplastin, such, in our hands, has been found unnecessary, provided no major change has been made in the preparation of the thromboplastin. We have been using the same standard conversion curve continuously for the past fourteen months. Indeed, there is no significant difference between more recently constructed conversion curves and those we prepared three years ago.

It appears[4, 12, 19-21] that the complexities of thromboplastins and plasmas, each with variable coagulation factors present in their make-up, together with various diluents and daily fluctuations of thromboplastic potency are among the more important and logical explanations for the differences in results obtained by use of the usual type activity curves and the present state of confusion between some laboratories. There is no question that such curves derived under these diverse conditions will result in markedly different assays of prothrombin activity. It is to be hoped that a "standard conversion curve" may help to eliminate some of these difficulties and make available to the clinician prothrombin activity estimations that will be easily verified.

SUMMARY

By application of a standard conversion curve and fresh normal human plasmas, the use of rabbit brain, rabbit lung or human placental thromboplastin has been found satisfactory for routine prothrombin determinations even though the thromboplastin had deteriorated either slightly or to a marked degree.

It is to be stressed, however, that an important requirement when using deteriorated thromboplastin with the standard conversion curve is the need of either a normal plasma (known by previous determinations) or two presumed normal plasmas. In order to allow a wide margin of safety with this method it is suggested that no thromboplastin be used that has deteriorated more than double its usual clotting time.

We are grateful to Mrs. Jeanne Hawk, R.N., and Mrs. Betty Krueger, R.N., for technical assistance in the collection of blood samples, and also gratefully acknowledge the valuable criticisms and suggestions of Dr. Clyde G. Culbertson.

REFERENCES

1. Discussion: Transactions of the 2nd Conference on Blood Clotting and Allied Problems. New York, 1949, Josiah Macy, Jr., Foundation, pp. 51-83.
2. Milstone, J. H.: The Chain Reaction of the Blood Clotting Mechanism in Relation to the Theory of Hemostasis and Thrombosis, Blood **4**: 1290, 1949.
3. Owren, P. A.: The Coagulation of Blood, Investigations on a New Clotting Factor, Acta med. Scandinav. Supp. 194, 1947.
4. Ware, A. G., Murphy, R. C., and Seegers, W. H.: The Function of Ac-Globulin in Blood Clotting, Science **106**: 618, 1947.
5. Frommeyer, W. B., Jr.: Determination of Prothrombin by the Dilution Method. Stability and Activity of Human and Bovine Prothrombin-Free Plasma, J. LAB. & CLIN. MED. **34**: 1356, 1949.

6. Ferguson, J. H., Travis, B. L., and Gerheim, E. B.: The Activation of Prothrombin, with Special Reference to "Thromboplastic Enzyme" (Tryptase), Blood 3: 1130, 1948.
7. Seegers, W. H., and Ware, A. G.: Recent Advances in Our Knowledge of Prothrombin, Am. J. Clin. Path. 19: 41, 1949.
8. Quick, A. J., and Stefanini, M.: The Concentration of the Labile Factor of the Prothrombin Complex in Human, Dog, and Rabbit Blood; Its Significance in the Determination of Prothrombin Activity, J. LAB. & CLIN. MED. 33: 819, 1948.
9. Discussion: Transactions of the 2nd Conference on Blood Clotting and Allied Problems. New York, 1949, Josiah Macy, Jr., Foundation, pp. 29-50.
10. Quick, A. J.: The Hemorrhagic Diseases and the Physiology of Hemostasis. Springfield, Ill., 1942, Charles C Thomas, p. 65.
11. Mann, F. D., Hurn, M., and Magath, T. B.: Observations on the Conversion of Prothrombin to Thrombin, Proc. Soc. Exper. Biol. & Med. 66: 33, 1947.
12. Schilling, F. J., and DeNatale, A.: Naturally-Occurring Anticoagulants and Accelerator Substance in Human Blood, Am. J. M. Sc. 218: 70, 1949.
13. Quick, A. J.: On the Quantitative Estimation of Prothrombin, Am. J. Clin. Path. 15: 560, 1945.
14. Olwin, J. H.: The Significance of Different Methods for Prothrombin Estimation and Their Relative Values, Surg., Gynec. & Obst. 90: 423, 1950.
15. Quick, A. J.: The Hemorrhagic Diseases and the Physiology of Hemostasis, Springfield, Ill., 1942, Charles C Thomas, p. 313.
16. Gerber, C. F., and Blanchard, E. W.: Factors in the Lability of Thromboplastin Solutions, Fed. Proc. 9: 47, 1950.
17. Hurn, M., Barker, N. W., and Magath, T. B.: The Determination of Prothrombin Time Following the Administration of Dicumarol, 3,3'-Methylenebis (4-Hydroxycoumarin), with Special Reference to Thromboplastin, J. LAB. & CLIN. MED. 30: 432, 1945.
18. Rosenfield, R. E., and Tuft, H. S.: Estimation of Prothrombin Level from Prothrombin Time, Am. J. Clin. Path. 17: 405, 1947.
19. Conley, C. L., and Morse, W. I.: Thromboplastic Factors in the Estimation of Prothrombin Concentration, Am. J. M. Sc. 215: 158, 1948.
20. Discussion: Transactions of the 2nd Conference on Blood Clotting and Allied Problems. New York, 1949, Josiah Macy, Jr., Foundation, pp. 11, 29, and 201.
21. Stefanini, Mario: Conversion Factors and Accelerators in the Formation of Thrombin, Blood 6: 84, 1951.

AN IMPROVED EOSINOPHIL COUNTING SLIDE

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Bar Harbor, Maine

IN RECENT years there has been a tremendous increase in interest in the eosinophil cells of the blood. This has been particularly stimulated by clinical experiments in which these cells were incorporated into a test for adrenal cortical activity.[1, 2] Furthermore, in the adrenalectomized mouse, an eosinopenia over a three-hour period has been found to be a specific response to adrenal cortical hormones.[3, 4] This response has been incorporated into a bioassay for these hormones[4-6] as well as for ACTH.[7] These procedures necessitated accurate and rapid methods of determining the number of circulating eosinophils. Most of the initial studies with the assay were made using the blood film procedure in which total and differential counts were performed. Later direct chamber counts were made and found to be approximately ten times faster than the blood film procedure. Additional studies were also made to simplify and speed up the direct procedure, such as designing a special counting chamber and improving the methods of recording data. The design of the counting chambers used is very important in order to perform eosinophil determinations rapidly with maximum accuracy. This report contains a description of a counting slide which has been in use in our laboratories for over a year, replacing the Fuchs-Rosenthal Slide which was formerly used.

The following points were thought to be desirable on such a slide:

1. There should be as many pairs of chambers as possible on each slide. This eliminates the continual changing from one slide to another which greatly slows up counting, and this also makes possible a counting procedure which reduces subjective error. It is customary to load and count two chambers from each pipette of blood taken. With the two-chambered slide, both chambers would be filled with blood from the same pipette. With a four-chambered slide, two different samples of blood would be on each slide, and the counter could be instructed to count one sample and then the other alternately.

2. Each chamber should be as simple as possible. The design commonly used in most WBC chambers of subdividing each one square millimeter area into sixteen was satisfactory. However, it was greatly desired that the chamber be simplified so that only a single change in direction of motion was necessary during the counting of each chamber. It was felt that the ideal chamber for counting would have only two rows, since the more rows there are the greater the possibility of skipping rows and thus not counting the entire chamber. For example, the Fuchs-Rosenthal Chamber consists of four rows each with four 1 square millimeter ruled areas. This necessitates additional time to change

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from one row to another, and increases the possibility of not counting all the rows, especially when large numbers of chambers are counted in a short period of time.

3. Each chamber should consist of ten 1 square millimeter ruled areas. Inasmuch as the accuracy of the count is proportional to the square root of the number of cells counted, it was decided that in most cases one, two, or four chambers would be counted for each blood sample, depending upon the accuracy desired and the number of eosinophils in the blood sample. If the number of 1 square millimeter areas counted was ten or units of ten, the multiplying factor to convert cells counted to cells per cubic millimeter of blood was usually a whole number.

4. The depth of the chambers should be 0.2 mm. rather than 0.1 mm. The number of eosinophils in the blood is usually small, and the volume of the chamber should be as great as possible in order to include more cells.

5. The chambers should be spaced on one side of the slide. This allows one side of the slide to be used for handling without necessitating great care to avoid touching the cover glass. This spacing of the chambers also helps to avoid accidentally reversing the slide.

6. An identification number should be placed on one side of each chamber. This helps the counter (1) to orient himself quickly on the chamber, (2) to keep the chambers in proper order of counting, and (3) to recognize if the slide had been accidentally reversed. Inasmuch as the counts are usually made from left to right, the number should be placed on the right-hand side of the chamber so it would appear on the left when viewed through the microscope.

7. The outer edge of each chamber should be beveled approximately 7 degrees. This bevel has been found to be an aid in charging the chamber, but it also acts as a reservoir to replace any fluid that might evaporate before the slide is counted. This movement of fluid occurs at such a slow rate that the cells are not shifted from one part of the slide to the other.

A manufacturer of hemocytometers was consulted and a counting chamber was designed with consideration for the practical problems of manufacturing as well as the above points. A dozen of these slides were made up and have been in use for approximately one year. The specifications of the slide are shown in Fig. 1.

In practice, the following procedures have been used with these slides:

One or more WBC pipettes are filled from each blood sample, mixed with diluent, and two chambers loaded from each pipette.

A. When one person does all the counting, the first pipette is loaded into chambers 1 and 3. The second pipette is loaded into chambers 2 and 4. A tally counter is used and the reading recorded in a vertical column without turning the counter back to zero after each count. The count for the first chamber is recorded at the bottom of the vertical column, the second chamber is recorded above it, etc. After all four chambers have been counted, the difference between each reading is determined and alternately placed on each side of the column. The two figures on one side of the column can be added to obtain the total number of eosinophils counted from each pipette.

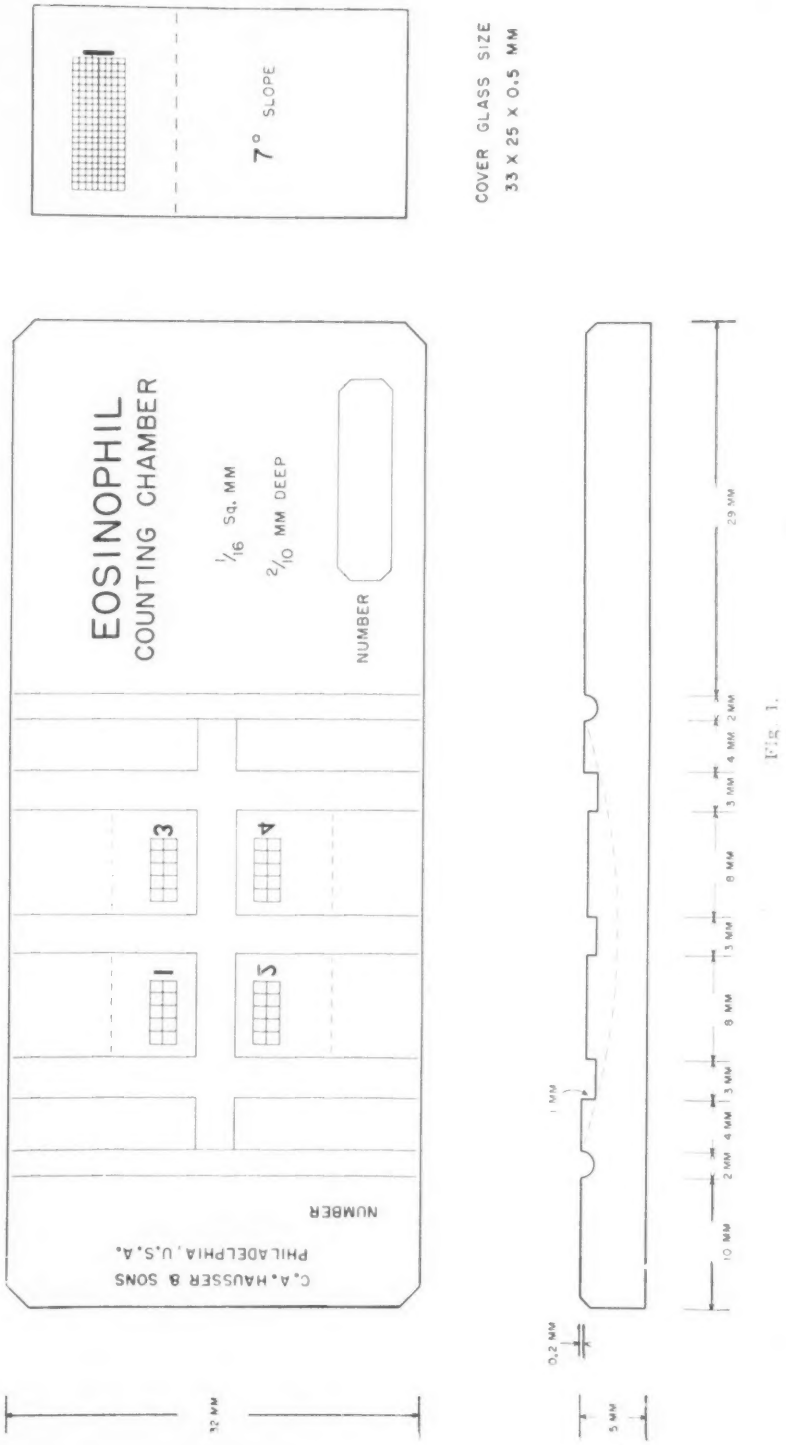
7° SLOPE
COVER GLASS SIZE
33 X 25 X 0.5 MM
EOSINOPHIL
COUNTING CHAMBER
1/16 SQ. MM
2/10 MM DEEP
NUMBER
1
2
3
4
C. A. HAUSSER & SONS
PHILADELPHIA, U.S.A.
NUMBER
32 MM
1 MM
0.2 MM
5 MM
10 MM
2 MM
4 MM
3 MM
8 MM
3 MM
8 MM
5 MM
4 MM
2 MM
29 MM

FIG. 1.

Example: Pipette A was loaded into chambers 1 and 3, and pipette B was loaded into chambers 2 and 4.

Data:

Chamber 4		65	
Chamber 3		54	11
Chamber 2	24	30	
Chamber 1		21	9
Initial Reading	21	0	
Total count	A = 45		B = 20

This system of counting has the advantage of reducing the subjective error involved when successive chambers loaded from the same pipette are counted. This is especially true when calculation of the cells counted per chamber is not performed until after the counting has been completed.

B. If two people are counting simultaneously, the chambers are handled in pairs and arranged in two rows. The first pipette is loaded into the first chamber of the first slide in each row. Simultaneous counts are then made.

Example: Four pipettes of diluted blood are loaded into two counting slides, one chamber on each slide from each pipette. The data were recorded as follows:

	COUNTER A		COUNTER B		TOTAL EOSINOPHILS PER 2 CHAMBERS
	TALLY	DIFFERENCE	TALLY	DIFFERENCE	
Chamber 4	75	32	80	32	64
Chamber 3	43	8	48	11	19
Chamber 2	35	19	37	23	42
Chamber 1	16	16	14	14	30
Initial tally reading	0		0		

The formula for calculating the number of eosinophils per cubic millimeter is:

$$\frac{\text{Number of eosinophils counted} \times \text{Dilution} \times 5}{\text{Number of 1 square millimeter areas counted}} = \text{Eosinophils per cubic millimeter}$$

For simplicity, the multiplying factor in the following chart may be used:

CHAMBERS COUNTED	DILUTION		
	1/10	1/20	1/100
1	5	10	50
2	2.5	5	25
4	1.25	2.5	12.5

The 1:10 and 1:20 dilutions are made with a WBC pipette, while the 1:100 solution is made with an RBC pipette.

Determination of error: The cells are distributed in a Poisson fashion in the counting chamber. This means that the error of counting is proportional to the square root of the number of cells counted, and is not less than 1 times the square root of the number of cells counted.[8, 11] However, in practice, with trained technicians an error of 1.2 times the square root of the number of cells counted has been obtained.[4]

The advice and technical assistance of J. E. Sullivan, L. E. Wragg, and K. Shaw is gratefully acknowledged, as well as splendid cooperation of C. A. Hausser and Sons, Philadelphia, Pa.

REFERENCES

1. Thorn, G. W., Forsham, P. H., Prunty, F. T., and Hills, A. G.: A Test for Adrenal Cortical Insufficiency, J. A. M. A. **137**: 1005, 1948.
2. Thorn, G. W., and Forsham, P. H.: Metabolic Changes in Man Following Adrenal and Pituitary Hormone Administration, Recent Progress in Hormone Research **4**: 229-288, 1949.
3. Speirs, R. S., and Meyer, R. K.: The Effects of Stress Adrenal and Adrenocorticotrophic Hormones on the Circulating Eosinophils of Mice, Endocrinol. **45**: 403-429, 1949.
4. Speirs, R. S., and Meyer, R. K.: A Method of Assaying Adrenal Cortical Hormones Based on a Decrease in the Circulating Eosinophil Cells of Adrenalectomized Mice, Endocrinol. **48**: 316-326, 1951.
5. Speirs, R. S., Wragg, L., Bonner, C. D., and Homburger, F.: A Seven Hour Bioassay for Urinary Corticoids, Proc. 2nd Clinical ACTH Conf. Blakiston Co., N. Y. **1**: 32-39, 1951.
6. Rosenberg, E., and Lewis, R. A.: Reduction in Eosinophil Level of Adrenalectomized Mice Following Injection of ACTH, Cortisone and Lipid Extracts of Human Urine. J. Applied Physiol. **3**: 164-172, 1950.
7. Speirs, R. S., and Panzenhagen, H.: Assay of ACTH Activity Based on Eosinopenic Response in Normal Mice. J. Clinical Endocrinol. (In preparation) 1952.
8. Berkson, J., Magath, T. B., and Hurn, M.: Error of Estimate of Blood Cell Count as Made with Hemocytometer, Am. J. Physiol. **128**: 309-323, 1940.
9. Best, W. R., and Samter, M.: Variation and Error in Eosinophil Counts of Blood and Bone Marrow, J. Hemat. **6**: 61-74, 1951.
10. Discombe, G.: Criteria of Eosinophilia. Lancet **250**: 195-196, 1946.
11. Rud, F.: The Eosinophil Count in Health and in Mental Disease, Acta Psychiat. et Neurol. Suppl. 40, 443 pp. 1947.
12. Speirs, R. S.: The Principles of Eosinophil Diluents, J. Hemat. In press, 1952.

A SIMPLE METHOD FOR REPEATED CARDIAC OUTPUT DETERMINATION IN THE UNANESTHETIZED DOG USING AN INDWELLING CARDIAC CATHETER

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METHODS utilizing the Fick procedure are generally acknowledged to be the most satisfactory for cardiac output determination. The major difficulty in chronic experiments has been in obtaining mixed venous blood simultaneously with the arterial samples and oxygen consumption data.

Both ventricular puncture[1] and the London cannula[2] have been used for this purpose, but we believe that these techniques are arduous and make difficult the maintenance of satisfactory basal conditions. It is probable that truly mixed venous blood is found only in the pulmonary artery, so that blood obtained elsewhere is of questionable significance.[3] Furthermore, a basal state in the unanesthetized dog is necessary for obtaining comparable serial cardiac outputs. These conditions are satisfied in the technique described below.

Polyethylene tubing or ordinary cardiac catheters were kept in place and patent, with their tips in the pulmonary artery, for as long as desired.

METHOD

Before catheterization dogs should be made accustomed to the arterial needling and to the mask* used for oxygen consumption. This is relatively simple when docile hounds are used.

Under intravenous pentobarbital anesthesia, an incision is made in the neck overlying the external jugular vein. A length of polyethylene tubing (about size PE 280†) or cardiac catheter (about No. 7) is inserted into the vein and passed under fluoroscopic control into the main pulmonary artery.‡ Polyethylene tubing has the advantage of a wide bore, but is more difficult to implant, being less flexible and not radiopaque. A metal plug at the tip, with the hole in the tubing just proximal, permits visualization of its passage. When in place, the catheter is filled with heparin and closed at its proximal end with a metal plug, or is bent double over a short distance and tied. The skin incision is then closed. Strips of adhesive tape are wrapped around the catheter where it emerges, and these are sutured into the skin. Adhesive tape and Ace bandage are then applied, firmly but not tightly, around the neck in such a way that by lifting one or two folds, the proximal end of the catheter can be brought to the surface.

For obtaining the daily cardiac output, the mask is applied and a resting oxygen consumption obtained. The plug is drawn from the tubing, a tight-fitting needle inserted, and the catheter is cleared by drawing out the heparin solution. A Luer-lok syringe is then used and the pulmonary and femoral arterial samples are obtained simultaneously. The catheter is then flushed with saline, refilled with heparin and the stylus is reinserted.

The animals should be maintained on penicillin (300,000 units intramuscularly) every other day.

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*For these experiments, a mask modelled after the Blalock mask[4] was used.

†Clay-Adams Co.

‡In placing the catheter fluoroscopically, care should be taken to leave no slack; otherwise, after several days the catheter tends to travel to the lung periphery and "pulmonary venous capillary" blood is obtained, or the catheter becomes occluded by the wall of a small blood vessel.

OBSERVATIONS

A representative normal dog provided the data shown in Table I. It may be seen that the figures vary from the average of 1.5 liters per minute by 10 to 15 per cent. This is the physiological variation one would expect during chronic determinations.

TABLE I. VARIATION IN CARDIAC OUTPUT OBTAINED ON A TRAINED DOG ON DIFFERENT DAYS*

DATE OF OBSERVATION	OUTPUT L./MIN.	CARDIAC INDEX L./MIN./M²
11-29-51	1.3	1.8
11-30-51	1.6	2.2
12- 4-51	1.4	1.9
12- 5-51	1.3	1.8
12-17-51	1.5	2.0
12-18-51	1.7	2.3
Average	1.5 ± 0.3	2.0 ± 0.4†

*Surface area obtained using nomogram based on formula S.A. $= \frac{2.27 \times L \times W^{.367}}{10{,}000}$

†S.D. $= \sqrt{\frac{\Sigma d^2}{n-1}}$.

There was no evidence of ill health or infection among the animals used.

There was no observed tendency for blood to diffuse from the pulmonary artery into the catheter between readings. If the catheter is flushed daily, or even every other day, it can be kept patent for two to four weeks, or longer, as desired.

At post-mortem, the sole lesions noted were occasional small mural thrombi in the right auricle. No valvular or pulmonary lesions have been found.

SUMMARY

A simple method is presented for obtaining serial cardiac output determinations in the dog. Catheters were passed through a jugular vein into the pulmonary artery, using fluoroscopic control. These were kept in situ and open for periods up to twenty-one days, permitting daily withdrawal of mixed venous samples from the main pulmonary artery.

We are indebted to H. Saiki for technical assistance while developing this method. We are grateful to Dr. L. N. Katz for valuable suggestions.

REFERENCES

1. Marshall, E. K.: Studies on the Cardiac Output of the Dog, Am. J. Physiol. **77**: 459, 1926.
2. Katz, L. N., and Steinitz, F. S.: Pulmonary Artery Pressure in Experimental Renal Hypertension, Am. J. Physiol. **128**: 433, 1940.
3. Warren, J. V., Stead, E. A., Jr., and Brannon, E. S.: The Cardiac Output in Man; A Study of Some of the Errors in the Method of Right Heart Catheterization, Am. J. Physiol. **145**: 458, 1946.
4. Blalock, A.: A Rubber Mask for Determination of O_2 Consumption of the Dog, J. LAB. & CLIN. MED. **12**: 378, 1926.

USE OF ION EXCHANGE RESINS IN PARTIAL PURIFICATION AND CONCENTRATION OF POLIOMYELITIS VIRUS

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A TECHNIQUE for the partial purification of the Lansing strain of poliomyelitis virus utilizing a strong base anion exchange resin (Amberlite XE-67) has been reported.[1] The purpose of this communication is to describe a procedure in which the preliminary elimination of extraneous material with a combination of anion and cation exchange resins makes possible the concentration as well as greater purification of the Lansing strain of poliomyelitis virus with Amberlite XE-67. In addition, a combination of ion exchange resins is introduced in the final procedure which circumvents the cumbersome process of dialysis. The advantages of the procedure are: (a) its capacity for handling large volumes of crude infected material in a relatively short period of time (three to four hours), and (b) its relative simplicity in that it can be conducted at room temperature without the use of a centrifuge.

MATERIALS AND METHOD

The Lansing strain of poliomyelitis virus from the nervous tissue of mice was used. A 10 per cent suspension of infected brains and cords in distilled water was stored at −26° C. for at least one week before use. More satisfactory adsorption of virus to Amberlite XE-67 was obtained from frozen stored material than from freshly prepared tissue suspension. All assays for virus were made by intracerebral inoculation in 4- to 6-week-old mice (CFW Albino Agouti strain, Carworth Farms). Ten mice were used for each dilution tested. A total of five ion exchange resins† were used in the procedure: Amberlite XE-67, a strong base anion exchanger, very finely powdered, used in the chloride form; Amberlite IRA-410, a strong base anion exchanger, bead size, used in the hydroxy form; Amberlite IR-120, a strong acid cation exchanger, bead size, used in the hydrogen form; Amberlite IR-4B, a weak base anion exchanger, bead size, used in the amino hydroxy form; and Amberlite IRC-50, a weak acid cation exchanger, bead size, used in the carboxylic form. The procedure is conducted in three distinct steps.

Step 1 involves preliminary removal of anions and cations from a 1 per cent CNS (uncentrifuged) tissue suspension of virus-infected material. One thousand milliliters of CNS tissue suspension in distilled water are passed through a monobed resin column prepared by making a one to one mixture of a strong base anion exchanger (Amberlite IRA-410) and a strong acid cation exchanger (Amberlite IR-120) which is placed in a glass column. Seven hundred fifty grams of this mixture is necessary for every liter of 1 per cent tissue suspension. The glass column is 4 inches in diameter and 5 feet long, fitted with a one-hole rubber stopper at one end in which is inserted a piece of glass tubing 3 to 4 mm. in diameter. Attached to this is a short piece of rubber tubing with a screw clamp to control the volume of flow. The

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resin bed is first washed with several volumes of distilled water and a water-head is allowed to remain. The 1 per cent virus tissue suspension is passed through the resin column as rapidly as possible. The column is then washed with sterile distilled water until the effluent becomes clear (approximately 3,000 ml.). These two effluents were kept separate to permit study of the effect of the resin mixture upon virus and the nitrogen content, but for practical purposes they can be combined.

Step 2 consists of adsorbing the virus containing material to Amberlite XE-67. Five grams of Amberlite XE-67 are mixed with each effluent from Step 1, or 10 Gm. to the combined effluents. The resin-tissue-virus mixtures are shaken by hand in stoppered bottles for twenty minutes, and then passed through a Buchner funnel with Whatman No. 1 quantitative filter paper. The resin from both effluents is collected on the same paper. As the extremely fine resin may pass through the filter paper during the initial phase of filtration the first filtrate is refiltered after a suitable resin bed has formed on the paper. The filtrate may or may not contain detectable virus. To be certain of obtaining all the virus, therefore, the filtrate is again treated with 2 Gm. of Amberlite XE-67. The sediment, consisting of resin-tissue-virus complex, is washed in a beaker with 40 ml. of 10 per cent disodium acid phosphate in distilled water for twenty minutes. This elutes the virus from the resin leaving the bulk of extraneous material on the resin. The virus-phosphate eluate is separated from the resin by filtration through the Buchner funnel.

In *Step 3* the excess disodium acid phosphate is removed from the virus-phosphate eluate. This can be accomplished either by dialysis against distilled water, or by the use of a monobed resin column. The resin column technique is simpler and less time consuming. A resin column that is satisfactory for this purpose consists of a 1 to 1 mixture of a weak cation exchanger (Amberlite IRC-50) and a weak anion exchanger (Amberlite IR-4B). One-half gram of resin mixture is used for each milliliter of virus-phosphate eluate. The resin mixture is placed in a 50 ml. burette and washed at a rapid rate with sterile distilled water until the effluent is clear. The virus-phosphate eluate is then passed rapidly through the resin column. The wetting volume of water in the column is measured, and an equal amount of the first effluent is discarded to prevent unnecessary dilution of the virus. If the effluent is amber in color, it is passed through the column a second time which renders it colorless. This effluent contains sufficient disodium acid phosphate to keep the virus in suspension (22 to 88 gamma of phosphorus per milliliter). Resin columns which remove the disodium acid phosphate completely from the eluate also remove the virus. The effluent is now ready for use. If it is to be used for intracerebral inoculation, it is tested in a few mice for possible irritant effects on the brain, perhaps from excess phosphate. If it is too irritating it can be passed through the resin again.

No attempt was made to sterilize the resins, but otherwise the experiments were carried out under sterile conditions. A modification[2] of the Conway procedure[3] was used for the determination of the total nitrogen content.

RESULTS

A total of five experiments were performed in which resins were used as described above to partially purify and concentrate the Lansing strain of poliomyelitis virus from the central nervous tissue of mice. The relative amounts of solutions and resins used in the several steps were varied in the first four experiments. Some degree of partial purification was achieved in each experiment, but in three the virus concentration was not increased more than fivefold and the virus yields did not exceed 12 per cent. The fourth experiment, however, in which the amount of resins and fluids were as described above in Methods, resulted in greater virus concentration and final yield. The fifth experiment represents an exact repetition of the fourth. The results of

TABLE I. PARTIAL PURIFICATION AND CONCENTRATION OF POLIOMYELITIS VIRUS FROM CNS-MOUSE TISSUE BY ION EXCHANGE RESINS

EXPERIMENTAL DATA					CALCULATIONS FROM DATA							
					INFECTIVITY							
EXP. NO.	VIRUS SUSPENSION	VOL. (ML.)	TOTAL N (γ/ML.)	LD_{50}* LOG-10	ACTIVITY /.03 ML. (ANTILOG OF LD_{50})	ACTIVITY PER ML.	SPECIFIC ACTIVITY PER γ N_2	TOTAL ACTIVITY	VOL. REDUCT (FOLD)	CHANGE IN VIRUS CONC. (FOLD)	CHANGE IN SPECIFIC ACTIVITY (FOLD)	VIRUS YIELD (%)
					x 1,000	x 1,000		x 1,000,000				
	Original 1% CNS	1,000	509	2.7	.5	17	33	17				
4	Final eluate	20	119	3.7	5	167	1,400	3	50	+10	+42	20
	Original 1% CNS	1,000	414	3.6	4	132	300	132				
5	Final eluate	40	94	4.6	40	1,320	14,200	52.8	25	+10	+47	40

In Experiment No. 4 final eluate was dialyzed. In Exp. No. 5 final eluate was resin treated, N values determined before passing virus-phosphate eluate through resin column.

*Method of Reed and Muench.[4]

the latter two experiments are shown in Table I. The initial and final volumes, the initial and final amounts of nitrogen per unit volume, and the initial and final virus titers (LD_{50} log of dilution) are shown. From these data the volume reduction, the change of virus concentration, the specific activity, and the virus yield were calculated. These two experiments resulted in tenfold increases in virus titer, and in forty-two and forty-seven fold increases in specific activity. The virus yields were 20 and 40 per cent, respectively.

The final nitrogen values in all five experiments did not exceed more than 0.34 to 1.0 per cent of the original total nitrogenous material.

DISCUSSION

The procedure described here is an improvement in several ways over the method previously described for the partial purification of poliomyelitis virus by means of a resin.[1] With the present procedure a tenfold *increase* in virus titer is obtained in contrast to a tenfold *decrease* in the previous procedure, a 20 to 40 per cent virus yield is obtained instead of only 5 per cent, while the amount of nitrogen associated with the final virus suspension represents only 0.3 to 1 per cent of the original total nitrogenous material in contrast to the 1.5 to 3.5 per cent obtained in the previous procedure.

SUMMARY

A new procedure is described in which the Lansing strain of poliomyelitis virus from the central nervous tissue of mice can be partially purified and concentrated solely with the use of ion exchange resins. This procedure has the advantages of being applicable to large volumes of crude material and of requiring only three to four hours and a minimum of apparatus.

REFERENCES

1. LoGrippo, G. A.: Partial Purification of Viruses with an Anion Exchange Resin, Proc. Soc. Exper. Biol. & Med. **74**: 208-211, 1950.
2. Luschinsky, H. L., and Singher, H. O.: Identification and Assay of Monamide Oxidase in the Human Placenta, Arch. Biochem. **19**: 95-107, 1948.
3. Conway, E. J.: Microdiffusion Analysis and Volumetric Error, New York, 1940, D. Van Nostrand Co., p. 75.
4. Reed, L. J., and Muench, H. A.: A Simple Method for Estimating Fifty Per Cent Endpoints, Am. J. Hyg. **27**: 493-497, 1938.

A SIMPLE METHOD FOR THE DETERMINATION OF TETRAETHYLTHIURAMDISULFIDE (ANTABUS) AND BLOOD LEVELS OBTAINED EXPERIMENTALLY IN ANIMALS AND CLINICALLY IN MAN

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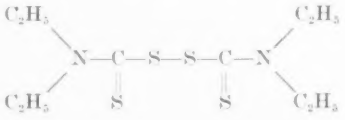
INTRODUCTION

ANTABUS is one of several chemical substances possessing the property of sensitizing biological organisms to the metabolic products of alcohol. Following the observations of Hald and associates[1] that subjects who had previously ingested the drug showed a variety of physiological disturbances upon the ingestion of ethanol, the compound was introduced as a possible adjunct in the revulsion treatment of chronic alcoholism. Since Antabus has been reported not to be excreted to any significant extent in either the urine or feces, and since in clinical trials it is ingested daily in quantities of one-half to one gram, there exists the possibility of an accumulation of Antabus or its metabolic products in body tissues. Early in our experimental studies with this agent it became apparent that it would be desirable to have a method for determining whether patients were receiving adequate blood levels of the drug, and whether accumulation of Antabus did occur as measured by its appearance in the blood stream. The following report will discuss the method devised in this laboratory for the determination of Antabus, the levels of the blood which have been found to occur in patients undergoing chronic therapy, and the blood levels found in experimental animals which have received a range of doses of the compound.

METHODS AND MATERIALS

Antabus is a thiuramdisulfide with the following structure:



This molecule contains two double-bond sulfur atoms in near juxtaposition, and consequently possesses considerable resonance. Therefore, it might be expected to present a well-defined

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absorption spectrum in the near ultraviolet region. A linear relationship was found to exist between the concentration of Antabus in a number of solvents and the optical density when measured at 285 mμ.

Hine and Divatia[2] previously reported that it is not possible to extract Antabus quantitatively from fresh whole blood or plasma. However, Antabus added to fresh blood or plasma can be quantitatively extracted upon the addition of cupric ions. Cupric ions form a pale yellow-colored complex with Antabus which is soluble in ethylene dichloride. The formation of this copper complex with thiuramdisulfide had previously been reported by Buchan and Rae.[3] Thiuramdisulfide has been used for some time in spot testing of copper where the characteristic yellow color can be seen in the visual spectrum. Recently Ovenston and Parker[4] have reported on an ultraviolet absorption technique for the determination of copper using diethyl dithiocarbamate as the characterizing agent. Extraction of the copper-Antabus complex with ethylene dichloride and the subsequent measurement of the optical density of this complex at wave length 270 mμ is the basis for the following method.

Factors Influencing the Extraction of Antabus From Blood.—During the preliminary investigation of procedures which could be utilized in the extraction of Antabus from the blood, it was observed that relatively greater concentrations of Antabus could be extracted from older blood than from freshly drawn beef blood. A time-recovery experiment was carried out in which the same concentration of Antabus, 10 μg per milliliter, was added to beef blood and plasma. Antabus was added to different aliquots of a refrigerated, unpreserved sample of beef blood and plasma on alternate days for a period of twenty-nine days, and recovery was attempted from the whole blood, from freshly centrifuged plasma, and from the previously separated plasma. The optimum recovery was reached at the end of the seventeenth day when 88 per cent of the added Antabus could be recovered from the whole blood and 97 per cent from the plasma. Following this there was again a progressively decreasing recovery of the added Antabus (Table I).

TABLE I. PERCENTAGE RECOVERY OF ANTABUS WITH INCREASING AGE OF BLOOD AND PLASMA

	FLUID		
DAY OF EXPERIMENT	BLOOD	FRESHLY CENTRIFUGED PLASMA	OLD PLASMA
1	33	43	50
3	37	37	51
5	37	37	55
9	42	43	80
11	50	50	85
13	66	63	89
15	78	79	93
17	88	82	97
21	78	44	88
25	57	41	83
29	46	25	63

Since it was felt that perhaps enzymatic destruction of the Antabus was taking place in the fresh blood, the influence of three general enzyme inhibitors was evaluated. Fluoride (0.2M), cyanide (0.1M), and silver nitrate (0.001M) were added to whole blood prior to the addition of Antabus. Antabus was added at varying times up to twenty-four hours after the addition of the enzyme inhibitors without influencing the percentage of recovery. The heating of whole blood, diluted equally with normal saline, at a temperature of 60° C. for fifteen minutes prior to the addition of Antabus did not increase recovery of this agent.

Attempted Chelation.—Since some disulfur compounds are known to form chelates with metallic ions, the effect of seven metallic ions on the chelation of Antabus was in-

vestigated. The substances which were added in 0.05M concentrations of the ions were the following: Co^{++}, Co^{+++}, Cu^{+}, Cu^{++}, Fe^{+++}, Fe^{++}, Mn^{++}, Mg^{++}, Ni^{++}, Ni^{+++}. None of these ions affected the absorption curve of Antabus with the exception of cupric copper. Following the addition of the cupric ion, there was a change in the general pattern of the absorption curve of the Antabus complex in ethylene dichloride, with a shift of the maximum points of absorption from the plateau commencing at 250 and extending to give a sharp peak at 285 (Fig. 1).

Following the observation that copper changed the absorption spectra of Antabus, a number of copper concentrations were tested, ranging from 0.001M to 0.1M final concentration. The maximum optical density of the copper-Antabus complex was obtained at a final copper concentration of 0.05M. However, it was found that this concentration when added to blood caused a precipitation of protein, and the final working concentration was chosen at 0.001 molar.

Extraction Time.—While the copper-Antabus complex could readily be extracted from aqueous solutions in five minutes, it was found that thirty minutes of shaking with ethylene dichloride was the shortest time in which quantitative extractions of the copper-Antabus complex could be made from whole blood.

Effect of pH.—The effect of three buffers, pH 5.3, pH 7.4, and pH 9.3, was tested on the recovery of Antabus. It was found that the recovery was approximately similar at all the three hydrogen ion concentrations. Because of the interfering effect of the dithiocarbamic acid which was discovered later, the pH 7.4 buffer was routinely used.

Description of the Procedure Finally Adopted for the Quantitative Determination of Antabus.—To one milliliter of whole blood or plasma in an all-glass extraction tube is added one milliliter of 0.002M copper sulfate solution, one milliliter of phosphate buffer, and 10 milliliters of redistilled ethylene dichloride. The tubes are shaken for a period of thirty minutes, centrifuged, and the upper aqueous layer removed with suction. An aliquot of the ethylene dichloride layer is transferred to a one-centimeter silica cell and the optical density determined on a Beckman DU spectrophotometer at wave lengths of 260 to 320 mμ. In the presence of Antabus the characteristic absorption curve of the copper-Antabus complex will be seen at a characteristic peak of 270 millimicrons (Fig. 1).

The difference in the optical density reading at 270 and 320 mμ is taken as a criterion of the concentration of Antabus in the sample. By utilizing the differences in optical density at the two wave lengths rather than the reading at 270 mμ alone, the small blank due to the interfering substances sometimes present in blood or plasma is eliminated. The concentration-optical density curve for the copper-Antabus complex is linear over a range of 5 to 200 μg per milliliter of Antabus. By this method recovery of Antabus added to whole blood or plasma averaged 100 ± 5 per cent over a wide range of Antabus concentrations.

Distribution of Antabus Between Red Blood Cells and Plasma.—It was of some interest to determine whether the Antabus in the blood would be carried predominantly in the red blood cells or in the plasma. In order to investigate this point, Antabus in a concentration of 100 μg per milliliter was added to whole blood and shaken for a period of ten minutes in order to obtain adequate distribution. An aliquot of the blood sample was then centrifuged, and determinations were made on whole blood and on plasma. It was found that, following the in vitro addition of Antabus, there was a nearly equal distribution between the plasma and the red blood cells. This experiment was repeated by administering Antabus in a dose of one gram per kilogram to each of three rabbits. Blood specimens were collected after thirty minutes and the whole blood and plasma analyzed. As in the in vitro experiment equal distribution of the Antabus between the red blood cells and the plasma was observed. Because only small blood samples were obtained from the patients, it was our custom to make determinations on whole blood.

Interfering Substances.—Ethylene dichloride extracts of fresh normal human blood in the presence of 0.002M copper sulfate give only a very small absorption in the wave length

region of 270 to 320 mμ. Therefore, there are apparently no interfering substances normally present in human blood. However, some difficulty may be experienced if either heparin or sodium fluoride are used as anticlotting agents. Heparin, even in very low concentrations, has an absorption spectrum with a characteristic peak at 272 millimicrons. This peak may be mistaken for copper-Antabus complex. It will also be observed that sodium fluoride will in the presence of copper give an absorption in this wave length which is somewhat above that of the normal background, and for that reason we have found that sodium oxalate is the most useful anticoagulant.

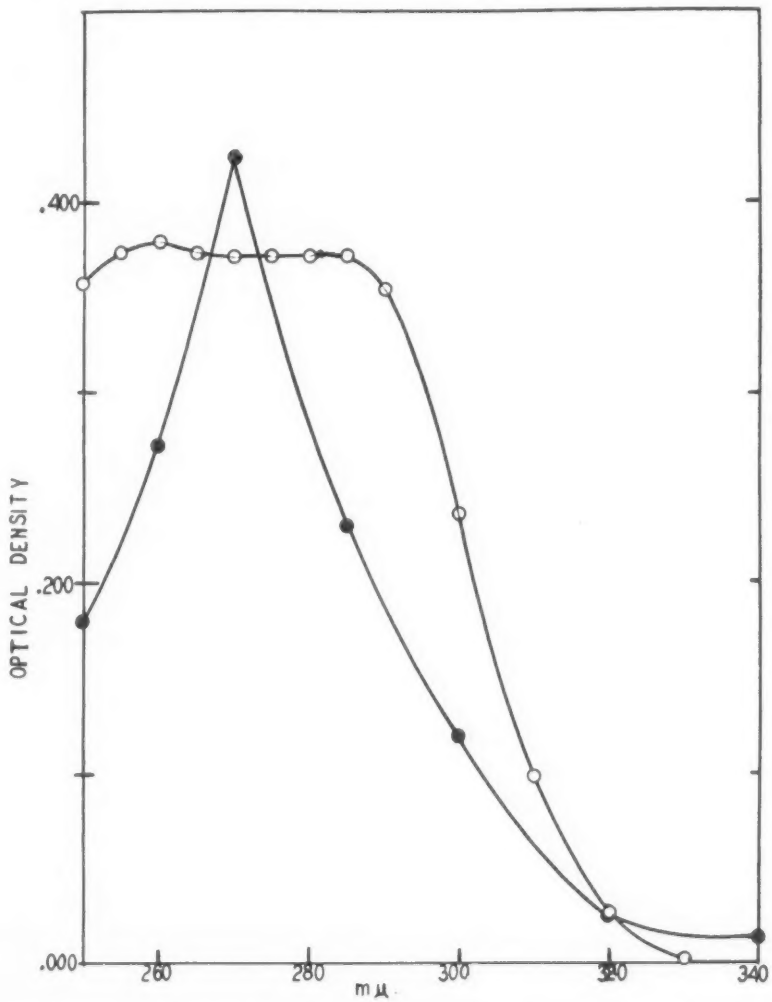

Fig. 1.—The absorption curve of Antabus and an Antabus-copper complex in ethylene dichloride. ○—○—○ Antabus; ●—●—● Antabus-copper complex.

If the Antabus molecule is split at the sulfur-sulfur linkage, one obtains two molecules of the diethyl dithiocarbamic acid. Due to the possibility that such a cleavage might take place in vivo with the formation of the diethyl dithiocarbamic acid, it was necessary to determine whether or not this substance would interfere in the determination and whether or not it was present in the blood of patients and experimental animals. The absorption

curve of the acid derivatives was, therefore, measured in ethylene dichloride in the presence of cupric ions, and it was found that the two curves, copper-Antabus complex and copper diethyl dithiocarbamate, were nearly similar and equal at equimolar concentrations. It was discovered, however, that the cupric salt of the acid was only partially extracted at pH's above 7, and a differentiation could be made between the compounds by extracting at the higher pH. Solutions of Antabus and the sodium salt of diethyl dithiocarbamic acid were made up in concentrations of 100 μg per milliliter and were extracted by the copper technique at three different buffers. There was a 50 per cent decrease in the optical density reading obtained with the acid derivative following an increase in pH, while the optical density of the Antabus was found to remain almost constant. Therefore, by extracting at both alkaline and acid pH's it was possible to establish the presence of the acid derivative. Blood obtained from patients and from rabbits having received high doses of Antabus when examined by this technique showed a constant optical density reading at all three pH's; and it can, therefore, be assumed that the diethyl dithiocarbamic acid is not present in the blood as a metabolic product of Antabus and will not be mistaken for this latter compound.

Several other compounds of therapeutic interest exhibiting absorption spectra in this wave length might conceivably interfere with the quantitative determination of Antabus. The thiobarbiturates, some of the sulfa derivatives, and salicylates give absorption in this region of the spectrum. If these compounds are suspected to be present, it would be advisable to eliminate their interference by extracting at the pH 9.3 where the Antabus will be extracted from the aqueous medium, but the other compounds will remain in solution.

EXPERIMENTAL

Blood Levels of Antabus in Rabbits.—The rate of disappearance of Antabus from the blood of rabbits receiving the compound intragastrically and the relationship between the dosage and the blood levels obtained were studied in the following experiments.

Six male rabbits weighing 3 ± 0.2 kg. were divided into three groups of two each and were administered Antabus in doses of 0.75, 0.50, and 0.25 Gm. per kilogram. Five milliliters of blood were withdrawn by cardiac puncture at varying time intervals (Table II) and the blood levels determined by the copper ethylene dichloride extraction technique, described earlier. At each dose level, the highest blood concentration was obtained at the 30-minute interval, and in no case was Antabus detectable after one hundred and twenty minutes.

A further study of the relationship between dosage and blood level obtained after a single administration of Antabus was obtained in the following experiment.

Three groups of three rabbits each were given Antabus in amounts of 1.0, 0.25, and 0.10 Gm. per kilogram intragastrically. The blood levels were determined after thirty minutes (Table III). It is of interest that no detectable quantity of Antabus could be found in the blood of the group which received 0.10 Gm. per kilogram.

A third experiment was carried out with four rabbits, each of which received a dose of 0.5 Gm. of Antabus per kilogram daily for seven days. The blood level was determined daily, thirty minutes after administration of the drug, and on the last day one hour prior to taking the blood specimens. The results of this

experiment appear in Table IV. It will be noted that only in the case of rabbit No. 1 was there any indication of a slight increase of Antabus in the blood following the repeated administrations. This increase probably should be interpreted as indicating an accumulation of Antabus in the blood, since there was no appreciable quantity of Antabus detectable in the blood of the animals prior to the administration of the last dosage.

TABLE II. MEAN BLOOD LEVELS (MICROGRAMS/MILLILITER OF ANTABUS IN RABBITS AT VARYING INTERVALS AFTER THE SINGLE ADMINISTRATIONS

TIME (IN MINUTES) AFTER ADMINISTRATION	DOSAGE OF ANTABUS ADMINISTERED INTRAGASTRICALLY		
	0.75 GM./KG.	0.50 GM./KG.	0.25 GM./KG.
30	10.2	5.7	4.1
60	4.4	3.2	1.0
120	0	0	0
140	0	0	0

TABLE III. RELATIONSHIP BETWEEN THE DOSAGE AND BLOOD LEVELS OF ANTABUS IN RABBITS THIRTY MINUTES AFTER A SINGLE ADMINISTRATION

DOSE OF ANTABUS (GM./KG.) ADMINISTERED INTRAGASTRICALLY	BLOOD LEVELS (Y./ML.) OBSERVED 30 MINUTES AFTER ADMINISTRATION OF ANTABUS
1.0	30 ± 3
0.25	4 ± 1
0.10	0

TABLE IV. THE EFFECT OF PROLONGED ADMINISTRATION OF ANTABUS ON BLOOD LEVELS IN RABBITS GIVEN A DOSE OF 0.5 GM./KG. DAILY FOR 7 DAYS

NO. OF DOSES	BLOOD LEVEL IN MICROGRAMS PER MILLILITER			
	RABBIT 1	RABBIT 2	RABBIT 3	RABBIT 4
1	10	15	10	12
2	12	15	11	12
3	15	15	12	12
4	15	17	10	11
5	16	15	10	10
6	17	15	12	12
7	16	15	13	13

The In Vitro Disappearance of Antabus from Fresh Human Blood.—We thought it of some importance to determine the stability of Antabus in fresh human blood, inasmuch as the time between the drawing of the sample and the determination might be of importance. Therefore, an experiment was conducted in which Antabus was added to fresh oxalated human blood in a concentration of 100 μg per milliliter. The blood was incubated at 37° C., and serial determinations were made on the blood specimen over a period of seventy-two hours. There was no appreciable change in the Antabus concentration of the blood up to forty-eight hours. After sixty hours, the concentration had decreased to 75 per cent, and at the end of seventy-two hours only 58 per cent of the original Antabus was present.

Antabus Blood Level in Patients Undergoing Antabus Therapy.—Antabus blood levels were determined on a series of twenty patients who had been re-

TABLE V. BLOOD LEVELS OF ANTABUS OBTAINED IN PATIENTS UNDERGOING CHRONIC THERAPY*

PATIENT	DAILY DOSE (GM.)	TIME UNDER THERAPY (WEEKS)	ANTABUS BLOOD LEVEL (MG. PER CENT)
B. B.	0.25	26	0.2
B. J.	0.50	4	0.6
	0.75	6	0.5
C. R.	0.50	25 (irregular)	0.15
		26	0.5
		29 (irregular)	0.0
C. U.	0.50	12	0.2
		16	0.5
C. W.	0.25	12	0.0
G. H.	0.50	10	0.8
		14	0.5
K. J.	0.25	11	0.15
K. G.	0.50	28 (off therapy 4 weeks)	0.0
L. C.	0.25	21 (irregular)	0.6
	0.50	26	0.45
M. E.	0.25	6	0.3
		7	0.9
		8	0.9
M. L.	0.5	34	0.0
M. R.	0.50	20	0.2
N. A.	0.5	6	0
		9	0.4
O. J. A.	0.5	26	0.4
O. J. E.	1.0	4	0.0
P. H.	0.50	5	0.0
P. L.	0.5	5	0.0
S. G.	0.50	3	0.1
	0.50	5	0.4
	0.25	7	0.2
	0.50	9	0.4
S. V.	0.5	5	0.0
	1.0	7	0.0
W. J.	0.5	2	0.4

*Patients under the supervision of Dr. A. Simon and Dr. E. A. Macklin.

ceiving Antabus in varying doses for a period of from three to thirty-four weeks. The patients described in this study belonged to the outpatient group which reported into the Langley Porter Clinic for physical, psychiatric, and laboratory examinations at assigned intervals during the year 1950. In all instances, the maintenance dose was taken by mouth from two to four hours prior to drawing of the blood sample. Thirteen of the twenty patients had detectable levels of Antabus in their blood at some time during the examination. The number of determinations on the patients varied from one to four during this course of therapy. In eight of the patients more than one determination was performed. The highest blood level, 0.9 milligrams per cent, was obtained in patient M. E., who had been on the lowest dose administered, 0.25 Gm., for only eight weeks. The mean blood level of patients at the twenty-sixth week was 0.39 milligrams per cent. There was no apparent correlation between the quantity of the drug taken and the blood levels of Antabus obtained when patients were compared with other patients on different dose levels or with themselves. In no case was there any indication of the occurrence of an accumulation of Antabus in the tissues of the patients as reflected by blood analysis. None of the patients on this regime showed any indication of toxicity

during the course of Antabus therapy other than mild complaints such as gastroenteric upsets, mild fatigue, increased weakness, and a general haziness and depression when first receiving the agent (Table V).

DISCUSSION

From the data obtained experimentally in animals and following blood analysis on patients receiving Antabus therapy, it is evident that Antabus is metabolized rather rapidly in the mammalian body, and that the opportunity for intoxication due to accumulation of Antabus per se in the tissues is rather remote. While the experiments of Hald and co-workers[1] indicated that a good portion of Antabus is not absorbed from the rabbit intestine even when given in large amounts, it has been our experience that the compound is absorbed through the gastrointestinal tract with a fair degree of rapidity, since peak concentrations of the drug following intragastric administration were reached within a period of thirty minutes. Unfortunately, the urinary excretion could not be studied in these experiments because of the inability to eliminate completely the blank from rabbit and human urine that interferes with the determination; therefore, the route of excretion is not clearly established. However, when an ethylene dichloride extract is made of urine which contains added copper and phosphate buffer, the characteristic yellow color will appear in the extract when the Antabus concentration is as little as 100 μg per milliliter. We have examined the urine of ten patients who have been on chronic Antabus therapy and have failed to detect the presence of any measurable amount of Antabus. If a patient receiving 0.5 Gm. of Antabus daily were to excrete only 20 per cent of the Antabus in the urine, it would be present in the concentration of approximately 100 μg per milliliter assuming 1 liter total daily output, and would be detectable by this method. Therefore, we can conclude that if Antabus is excreted at all by way of the urinary tract, not more than 20 per cent of the drug will leave the body by this route.

Although there was no apparent correlation between the dosage and the blood level obtained in patients, the animal experiments indicated that in general a higher blood level could be expected with increase in dosage, at least for a short period of time.

It was hoped that a method could be devised which would aid the clinician in determining whether or not the patient was taking his Antabus as prescribed, since alcoholics are unreliable in conscientiously taking any type of medication. Due to the lack of any significant blood level, more than a few hours after the administration of the drug, a blood analysis is only occasionally of value. Additional confirmation to the lack of correlation between blood level and ingestion was obtained among certain of our inpatients who failed to show measurable blood levels of the drug when the ingestion of the drug had been carefully ascertained.

SUMMARY

A quick reliable spectrophotometric method for the determination of Antabus in whole blood and plasma has been developed.

Antabus disappears rapidly from the blood stream of rabbits who have been administered doses as high as the LD_{50} (1 Gm. per kilogram).

There is no correlation between blood levels of Antabus and the amount of drug ingested.

There is apparently no accumulation of Antabus in the tissues of patients undergoing Antabus therapy.

Acknowledgment is made to the Ayerst, McKenna & Harrison Limited, New York, N. Y., for the Antabus used in this study.

REFERENCES

1. Hald, J., Jacobsen, E., and Larsen, V.: The Sensitizing Effect of Tetraethylthiuramdisulphide (Antabus) to Ethylalcohol, Acta pharmacol. et toxicol. **4**: 285-297, 1948.
2. Hine, C. H., and Divatia, K. J.: A Simple Spectrophotometric Method for Determination of Tetra-ethyl Thiuramdisulfide (Antabus) in Blood and Urine, Fed. Proc. **9**: 286, 1950.
3. Buchan, S., and Rae, W. D.: Chemical Nature of the Rubber-to-Brass Bond, Tr. Institute Rubber Industry **21**: 323-342, 1946.
4. Ovenston, T. C. J., and Parker, C. A.: Notes on the Spectrophotometric Determination of Copper as Diethyldithiocarbamate, An. Chem. Acta **4**: 135-150, 1950.

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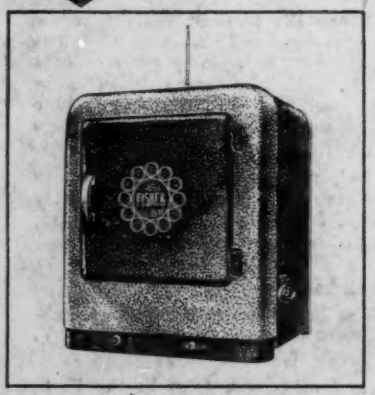

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